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REMARKS

Claims 5-17 are active. Claims 1-4 and 18-23 have been withdrawn from consideration. Claim 5 has been amended to refer to a consensus NF-κB binding site. Support for this amendment is found in the specification on page 4, last four lines. Minor changes have been made to claims 3 and 15 and claim 10 has been further limited. Accordingly, the Applicants do not believe that any new matter has been added.

The Applicants thank Examiner Falk for the courteous and helpful interview of December 14, 2004. To address the description rejection, it was suggested that claim 5 include a structural limitation on the compound employed. It was suggested that scientific publications showing that a representative number of brain diseases were associated with NF-κB activation would help address the enablement rejection. Minor revisions to address the indefiniteness rejection were also discussed. The Applicants have now revised claim 5 to include a structural limitation, present additional scientific publications showing that the NF-κB was well-known and that a representative number of brain diseases are associated with NF-κB. Favorable consideration is respectfully requested.

Election/Restriction

The Applicants acknowledge that the restriction requirement has now been made FINAL.

Rejection—Double Patenting

Claims 5-17 were rejected under the judicially-created doctrine of obviousness-type double patenting as being unpatentable over claims 5, 6, 8, 9, and

12-23 of allowed U.S. Application No. 09/466,913. The Applicants respectfully request that this rejection be held in abeyance pending the identification of otherwise allowable subject matter.

Rejection—35 U.S.C. §112, first paragraph

Claims 5-17 were rejected under 35 U.S.C. 112, first paragraph, as lacking adequate description of particular structural limitations on the compound employed. Independent claim 5 has now been amended to require the compound to comprise a NF-κB consensus binding site addresses part of this rejection. The consensus sequence of the NF-κB binding site. This limitation is described in the specification on page 4, last four lines.

The specific NF kappa motif sequence (5'-GGGACTTTCC-3') and the general consensus sequence (5'-GGGRNNYYCC-3') were well-known as of the filing date as shown by the attached documents:

Schreck et al., Nucleic Acids Res. 18(22):6497 (1990), see abstract;

Zabel et al., J. Biol. Chem. 266 :252 (1991), see abstract;

Ranganathan et al., Nuc. Acids Res. 21:1959 (1993), see abstract.

Xie et al., J. Biol. Chem. 269 :4705 (1994), see page 4705, col. 2;

Lenardo et al., Cell 58:227 (1989), see page 229;

Grilli et al., Int. Rev. Cytol. 143:1 (1993), see page 19,

Liou et al., Curr. Opin. Cell Biol. 5:477 (1993), see page 484; and

Siebenlist et al., Annu. Rev. Cell. Biol. 10:405 (1994), see page 407.

Accordingly, the Applicants respectfully submit that this description rejection may be withdrawn.

Rejection—35 U.S.C. §112, first paragraph

Claims 5-17 were rejected under 35 U.S.C. 112, first paragraph, as lacking adequate enablement for NF-κB inhibitors other than those comprising SEQ ID NO: 1 and a liposomal delivery system for treatment of cerebral vasospasm associated with subarachnoid hemorrhage. The Applicants submit that one with skill in the medical or molecular biological arts would understand how to target an NF-κB decoy to the brain, for example, as described at the bottom of page 10 of the specification and as exemplified on pages 11-12. Thus, undue experimentation would not be required to select an appropriate vehicle or route of administration to appropriate target the nucleic acid or nucleic acid analog to the appropriate tissue.

The claimed method employs a compound that comprises the NF-κB consensus binding site as a NF-κB decoy. Page 3, last paragraph, of the specification discloses that such decoys are particularly effective for use in brain tissues against disorders caused by unwanted activation of genes regulated by the NF-κB transcription factor and the top of page 4 describes a number of such diseases. To show the role of NF-κB in particular brain diseases, the Applicants direct the Examiner's attention to the following scientific publications:

(1) Alzheimer's Disease:

Brain Res. 735:159 (1996). The results suggest that enhanced expression of neuronal NF-κB occurs in areas affected to Alzheimer's disease pathology.

PNAS 94:2642 (1997). Activation of NF-κB was observed in neurons and astrologia of brain sections from subjects with Alzheimer's disease.

(2) Cerebral infraction:

Brain Res. 739: 343 (1996). These results suggest that enhanced expression of astrocytic NF-κB occurs in cerebral infracted areas.

(3) HIV-1 encephalitis:

Neuropathol. Appl. Neurobiol. 21:518 (1995). High-level activation of NF-κB may promote the sustained production of neurotoxins by microglia and macrophages during HIV-1 encephalitis.

(4) Head trauma:

Neurosci. Lett. 197:101 (1995). These data indicate that traumatic brain injury (TBI) produces transient increases in NF-κB DNA binding activity.

(5) Convulsant-induced seizures:

Neurosci. Lett. 170:145 (1994). Nuclear factor κB in rat brain: enhanced DNA-binding activity following convulsant-induced seizures.

In view of the above scientific references, the Applicants submit that one with skill in the art would be enabled to use the claimed method without undue experimentation for the treatment of brain disorders and diseases. Accordingly, the Applicants respectfully request that this rejection be withdrawn.

Rejection—35 U.S.C. §112, second paragraph

Claim 15 was rejected under 35 U.S.C. 112, second paragraph, as being indefinite. This rejection is moot in view of the amendment above. The term "unit" is described in the specification on page 5, lines 10-11. This term refers to a sequence comprising one or more repeats a particular oligonucleotide sequence, e.g., tandem repeats of SEQ ID NO: 1.

CONCLUSION

In view of the above amendments and remarks, the Applicants respectfully submit that this application is now in condition for allowance. Early notification to that effect is earnestly solicited.

Respectfully submitted,

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NF-κB and Rel: Participants in a Multiform Transcriptional Regulatory System

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I. Introduction

Molecular regulatory pathways were originally identified as series of covalent enzymatic modifications of substrate molecules during intermediary metabolism. Recent gene regulation research has revealed subtle and complex molecular regulation by information pathways involving noncovalent interactions between macromolecules. High affinity interactions between proteins with DNA sequences play a key role in governing gene transcription. Regulatory interactions also occur between transcription factors and RNA polymerases. In an increasing number of examples, protein–protein associations between various types of transcriptional regulatory factors determine the specificity of gene activation. These noncovalent information pathways are also intimately interlaced with enzymatic control points such as phosphorylation or other forms of post-translational modification. Together these pathways form a network that shuttles information throughout the cell allowing physiological needs to be met by selective gene expression.

Modulation of gene expression by the NF-κB/Rel family of protein complexes illustrates many of the newly emerging themes of intracellular regulation. Associations of the binding and regulatory subunits of NF-κB *operate at the center of a variety of different signal pathways.* NF-κB can process and integrate instructions that come from the extracellular milieu as well as detect intracellular events originating in either the cytoplasm or the nucleus. NF-κB then transmits this information with astonishing rapidity to the transcriptional machinery by directly binding to a range of different DNA sequences in gene control regions. NF-κB comprises members of a family of dimer-forming proteins with homology to the *rel*

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oncogene. Its binding, location in the cell, and transcriptional properties are regulated by a second family of proteins that contain repeats of a domain characteristically found in the erythrocyte protein ankyrin. The DNA sequences which bind NF-κB are also recognized by a third family of structurally distinct proteins bearing the "zinc-finger" DNA interaction domain. This highly evolved system controls a wide diversity of genes and thus provides an interesting paradigm for several areas of molecular and cellular research.

II. NF-κB Function in B Cells

In 1986, Sen and Baltimore first identified NF-κB as an apparently tissue-restricted factor that activated the Ig κ light chain intron enhancer during B-lymphocyte development. They used an electrophoretic mobility shift assay to detect a protein complex with a DNA sequence which was then called the B site (Sen and Baltimore, 1986a) (see Fig. 1). The B site was a 10-nucleotide DNA sequence in a region of the large intron of the κ light chain gene that had been defined by various functional assays as a B-cell-specific enhancer element (Queen and Baltimore, 1983; Bergman *et al.*, 1984). A hint to the importance of this sequence was that it was also present in the enhancer of the simian virus-40 (SV-40) DNA tumor virus (Emorine *et al.*, 1983; Sen and Baltimore, 1986a).

NF-κB had the striking appearance of being constitutively present only in B cells of the "appropriate" stage for Ig κ light chain gene expression (mature B cells and plasma cells, but not pre-B cells)(Sen and Baltimore, 1986a). Even more remarkable was that NF-κB was inducible in pre-B cells. For example, in the pre-B tumor line 70Z/3, NF-κB could be induced by agents such as lipopolysaccharide (LPS), active phorbol esters, cycloheximide, IL-1, tumor necrosis factor-α (TNF-α), and virus infection (Sen and Baltimore, 1986b; Atchison and Perry, 1987; Shirakawa *et al.*, 1989; Lenardo *et al.*, 1989). Because these same agents stimulated transcription of the κ locus, inducible NF-κB in pre-B cells strengthened the correlation with κ light chain gene expression (Nelson *et al.*, 1985). Chromatin changes and occupancy of the NF-κB site were directly demonstrated in intact nuclei from LPS-treated 70Z/3 cells (Gimble and Max, 1987; Hromas *et al.*, 1988). More recent studies have also found poor activation of NF-κB in genetic variants of the 70Z/3 cell line that display defective κ gene expression in response to LPS (Briskin *et al.*, 1990; Rooney *et al.*, 1990a,b).

The observation that cycloheximide induced NF-κB was especially prescient (Sen and Baltimore, 1986b). NF-κB induction apparently did not require new protein synthesis; in fact, cycloheximide and other stimulants caused a superinduction of NF-κB (Sen and Baltimore, 1986b). This correlated with the lack of a requirement for new protein synthesis for κ gene expression (Wall *et al.*, 1986) and led to the proposal that a preexisting form of NF-κB, which was controlled by a labile inhibitory protein, might reside in the cell prior to induction (Baltimore, 1987).

The functional role of NF-κB was demonstrated by mutational analysis of the κ intronic enhancer (Lenardo *et al.*, 1987) (see Fig. 2). Alterations

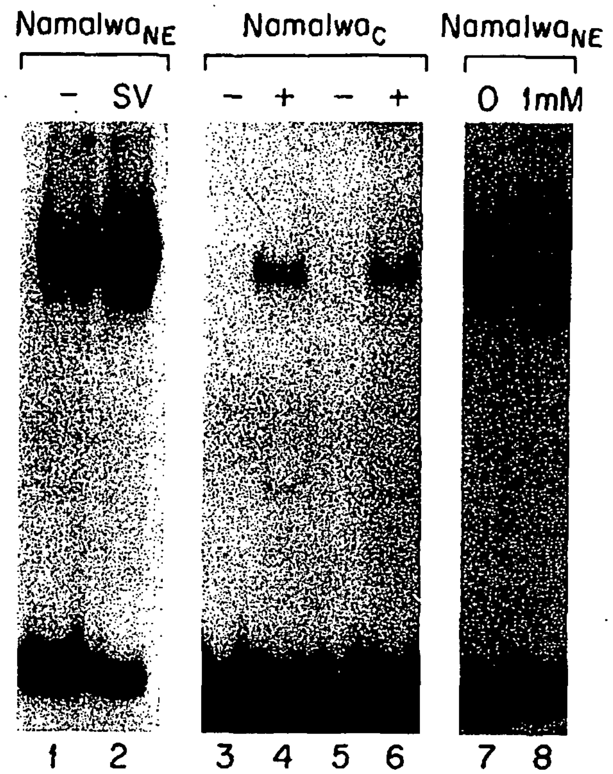


FIG. 1 Mobility shift electrophoresis analysis of extracts from Namalwa human mature B lymphocytes using the κB binding site from the Ig κ gene. Lanes 1 and 7 are nuclear extracts from unstimulated cells and lane 8 is the same with 1 m*M* GTP added to the binding reaction; lane 2 is nuclear extract from Sendai virus-induced cells. Lanes 3 to 6 are cytosolic extracts in which sodium deoxycholate treatment (+, lanes 4 and 6) has been used to free NF-κB from I-κB. These results and the methods for these experiments have been previously described in Lenardo *et al.* (1989).

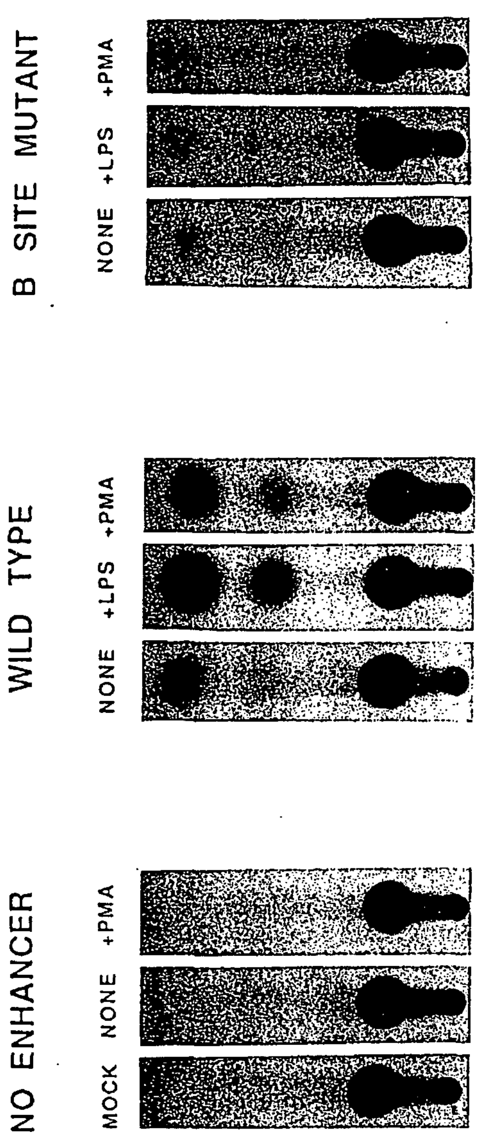


FIG. 2 Analysis of transcriptional activity conferred by the κB binding site in the Ig κ gene enhancer in mouse PD-31 pre-B lymphocytes. The left panel shows the bacterial chloramphenicol acetyltransferase activity for the truncated *c-fos* reporter plasmid with no enhancer. The middle panel shows the bacterial chloramphenicol acetyltransferase activity conferred by the 470-bp version of the Ig κ enhancer in unstimulated cells (none), or in cells stimulated with LPS or phorbol ester (PMA). The right panel shows the Ig κ enhancer with multiple point mutations in the κB binding site that eliminate binding of NF-κB in unstimulated cells (none), or in cells stimulated with LPS or PMA. These results and the methods for these experiments have been previously described in Lenardo *et al.* (1987).

of the B site that interfered with NF-κB binding destroyed both basal *enhancer function in mature* B cells and *inducibility in pre*-B cells. The mutated enhancer was completely inactive. Further experiments revealed that the NF-κB binding site on its own could act independently as a strong tissue-specific and inducible enhancer element (Pierce *et al.*, 1988; Wirth and Baltimore, 1988). In fact, two copies of an oligonucleotide containing only the NF-κB site stimulated transcription as well as the entire κ enhancer in transient transfection assays.

The initial conceptualization, based on the correlation to κ light chain gene expression, was that NF-κB was the key regulatory molecule for light chain gene activation. It was even imaginable that NF-κB orchestrated the entire transition from pre-B to mature B phenotype by turning on a set of differentiation genes. Four pieces of evidence have since suggested that the true picture is more complicated and more interesting. First, γ-interferon can activate κ gene expression and not alter the level of NF-κB binding activity in pre-B cells (Briskin *et al.*, 1988; Bomsztyk *et al.*, 1990). Second, the endogenous κ light chain gene has been found to be expressed at high levels in myeloma cell lines that contain little NF-κB and show no κ intronic enhancer activity (see below) (Atchison and Perry, 1987). Third, NF-κB does not seem to regulate the recently delineated enhancer elements in the Ig λ light chain loci (Hagman *et al.*, 1990). Fourth, as described below, elements other than the intron enhancer contribute to κ gene expression and do not involve NF-κB. Taken together, these findings would best portray NF-κB as one part of a transcriptional differentiation program that has multiple, perhaps sequential, but functionally similar elements. Further work will be needed to describe the interplay between the elements of this program and how they coordinate the maturation of pre-B to B cells.

One interesting paradox in Ig gene regulation that illustrates the complexity of eukaryotic gene analysis has been recently resolved. Early studies of the Ig heavy and light chain genes suggested that the intronic enhancer elements were dispensable for high level gene expression (Schaffner, 1988). As mentioned above, a myeloma line with little NF-κB was found to have robust Ig κ gene expression (Atchison and Perry, 1987). This led to the hypothesis that once the Ig genes were triggered, the enhancers and their associated binding proteins might not be needed for the maintenence of transcription (Klein *et al.*, 1985; Atchison and Perry, 1987). Preservation of transcription might be attained by the methylation status of the gene or particular chromatin configurations (Kelley *et al.*, 1988; Atchison and Perry, 1988). Evidence against this idea was that NF-κB was only rarely absent in mature cells of the B lineage (Sen and Baltimore, 1986a). Moreover, a continuous requirement for the enhancer element was found for the Ig heavy chain gene (Grosschedl and Marx, 1988). A coherent picture of Ig gene control subsequently emerged with the discovery of additional enhancer elements for both the Ig heavy chain and κ genes at significant distances downstream of the constant region (Meyer and Neuberger, 1989; Pettersson *et al.*, 1990). In the case of the κ gene, the downstream enhancer was stronger than the intronic enhancer and essential to achieve high enough expression of κ genes in transgenic *mice to cause allelic exclusion* (Meyer and Neuberger, 1989; Meyer *et al.*, 1990). Significantly, the downstream κ enhancer is not activated by NF-κB, indicating that multiple pathways lead to high level κ gene expression (Meyer *et al.*, 1990; M. Atchison, personal communication).

Although constitutively present in the nucleus, NF-κB can be further modulated in B cells. For instance, higher levels of nuclear NF-κB can be induced in human Namalwa cells by virus infection (Lenardo *et al.*, 1989). *At least part of the NF-κB that is induced in certain B cells seems to come* from the "masked" cytoplasmic form (see below). It also becomes evident in whole cell extracts from Daudi cells with a mild denaturant (Visvanathan and Goodbourn, 1989), or in mouse S194 myeloma cells by HTLV-1 *tax* gene product (Mauxion *et al.*, 1991). However, preexisting but nonbinding NF-κB does not appear to be present in all mature B cells (Baeuerle *et al.*, 1988). Recently, Marcuzzi *et al.* (1989) and Liu *et al.* (1991) reported *that in murine splenic B cells*, NF-κB, which is already present to some degree constitutively, can be induced further after Ig crosslinking.

NF-κB has been implicated in the transcriptional activation of a variety of genes other than the κ gene in B cells. These include the MHC class I and β2 microglobulin genes (Yano *et al.*, 1987; Baldwin and Sharp, 1988), as well as the genes for MHC class II and its associated invariant chain Ii (Blanar *et al.*, 1989; Pessara and Koch, 1990; Zhu and Jones, 1990), and other early activation genes (Anderson *et al.*, 1992). A motif similar to the consensus binding sequence of NF-κB has been found in the promoter of the gene for the murine B-cell tyrosine kinase *hck* (Lock *et al.*, 1990).

As in other cell lineages, NF-κB has to be viewed in B cells as an eclectic mediator of gene transcription that facilitates rapid genetic interpretation of extracellular signals. Two important regulatory issues are highlighted by the study of NF-κB in the B lineage. First, how does NF-κB turn on specific genes in the B lineage despite the fact that it induces transcription of many genes in many lineages? Second, how is NF-κB binding activity maintained constitutively in mature cells of the B lineage? One explanation for the B-cell-restricted response of certain genes to NF-κB has come from a study of the Ig κ intronic enhancer. Pierce *et al.* have shown that NF-κB's effect on the κ enhancer is under the influence of a negative element (1991). They identified a 232-base pair (bp) fragment located 5′ of the NF-κB binding site that makes the enhancer nonresponsive to NF-κB in non-B cells. Strikingly, this silencer element works in a distance- and orientation-independent fashion.

The issue of how B cells maintain a certain fraction of NF-κB in a nuclear binding form has not yet been adequately explained. There is some evidence that NF-κB is constitutively present in some T-cell lines and thymocytes (Baeuerle *et al.*, 1988; Bohnlein *et al.*, 1988; Leung and Nabel, 1988; Cross *et al.*, 1989; Lin *et al.*, 1990; Korner *et al.*, 1991) and possibly in mature macrophages (Griffin *et al.*, 1989). The mechanism for constitutive *activity in any cell lineage has not been determined.* Evidence from studies with the cloned genes indicates that the mRNAs for the NF-κB binding subunits p50 and p65 are not elevated as cells mature in the B lineage (Ghosh *et al.*, 1990; Nolan *et al.*, 1991). The simplest model would entail modulation of the level of the cytoplasmic inhibitor, I-κB, but the distribution and functional status of this molecule and its relatives have not yet been evaluated in different lineages.

III. NF-κB as an Inducible Transcriptional Activator

The property that gives NF-κB a widespread significance in cellular regulation is its role as a mediator of inducible gene transcription. The key features of NF-κB transcriptional control are that *it is fast and versatile* and is used in many different gene systems. It also has the important ability to carry signals from the cytoplasm into the nucleus and trans-activate specific genes by binding directly to their promoters.

The versatility of the NF-κB system is evident in the large number of agents able to up-regulate the levels of NF-κB binding in the nucleus. Unlike transcription factors which respond to a limited number of inducers such as the heat shock factor or specific steroid receptors, signals from a number of directions converge on NF-κB to be conveyed into the nucleus. Importantly, signals that pass through NF-κB appear to be launched from different parts of the cell (Lenardo and Baltimore, 1989; Baeuerle, 1991). They may emanate from cell surface molecules such as the receptors for cytokines (IL-1, TNF-α, and TNF-β), lectins, or LPS. They may come from elements of second messenger pathways that are associated with events at the cell membrane such as phorbol esters or calcium ionophores. They may arise in the cytoplasm through kinase induction by dsRNA or by inhibition of protein synthesis. They may also involve triggering by intranuclear proteins such as the Tax trans-activator of the HTLV-1 virus that induces NF-κB. *Finally*, and perhaps most surprisingly, induction may even be signaled at the DNA level such as by UV light and other agents that cause DNA damage. A list of the known inducing agents for NF-κB is given in Table I.

The kinetics of the NF-κB response to inducing agents indicate that it can be extremely rapid and quite sensitive. For example, early studies

TABLE I
Inducers of NF-κB

Inducer	Cell type	Species	Reference
Lipopolysaccharide	Pre-B (70Z/3; 3-1)	m	Sen and Baltimore, 1986b
	Splenic B	m	Atchison and Perry, 1987
	Fibrosarcoma	m	Liu *et al.*, 1991
	(WEHI 164)	h	Gromkowski *et al.*, 1990
	Monocyte/macrophage (mono-mac-6)		Haas *et al.*, 1990
Protein synthesis inhibitors	Pre-B (70Z/3)	m	Sen and Baltimore, 1986b
	Splenic B	m	Liu *et al.*, 1991
Phorbol ester	Pre-B (70Z/3)	m	Sen and Baltimore, 1986b
	T (Jurkat)	h	Sen and Baltimore, 1986b
	HeLa	h	Sen and Baltimore, 1986b
	Splenic B	m	Liu *et al.*, 1991
	Monocyte/macrophage	h/m	Griffin *et al.*, 1989
Anti-IgM	Splenic B	m	Marcuzzi *et al.*, 1989
		m	Liu *et al.*, 1991
HTLV-I *tax*	Plasmacytoma	m	Mauxion *et al.*, 1991
	T (Jurkat)	h	Leung and Nabel, 1988; and others (see text)
dsRNA	Osteosarcoma (MG63)	h	Visvanathan and Goodbourn, 1989
	HeLa		
	Fibrosarcoma (WEHI 164)	h	Visvanathan and Goodbourn, 1989
	Fibroblast L929	m	Gromkowski *et al.*, 1990
		m	Lenardo *et al.*, 1989
TNF-α	Pro-monocytic (U937)	h	Griffin *et al.*, 1989
	Rat-2 fibroblast	r	Pessara and Koch, 1990
	Myelomonocytic (WEHI 3B)	m	Pessara and Koch, 1990 Osborn *et al.*, 1989
	T (Jurkat)	h	Gromkowski *et al.*, 1990
	Fibrosarcoma (WEHI 164)	m	Banerjee *et al.*, 1989
	Hepatoma (HepG2)	h	
	Erythroleukemia (K562)	h	Meichle *et al.*, 1990
	Macrophages	m	Collart *et al.*, 1990
Lymphotoxin	Myelomonocytic	h	Hohmann *et al.*, 1990a, 1991b
Interleukin-1	T (YT)	h	Freimuth *et al.*, 1989
	T (Jurkat)	h	Osborn *et al.*, 1989
	T (EL-4-E1)	m	Novak *et al.*, 1990
	Foreskin fibroblast	h	Zhang *et al.*, 1990
Interleukin-1	Hepatoma (HepG2)	h	Nonaka and Huang, 1990
Interleukin-2	T (SPB21)	h	Hazan *et al.*, 1990

(*continues*)

TABLE I (*continued*)

Inducer	Cell type	Species	Reference
Phytohemagglutinin	T (Jurkat)	h	Sen and Baltimore, 1986b
	Pro-monocytic (U937)	h	Libermann and Baltimore, 1990
Concanavalin A	T (Jurkat)	h	Rattner *et al.*, 1991
Calcium ionophore	T (EL-4-E1)	m	Novak *et al.*, 1990
Anti-CD2	Monocytes	h	Bressler *et al.*, 1991
Anti-CD3	T (AR-5)	m	Jamieson *et al.*, 1991
	T (SPB21)	h	Hazan *et al.*, 1990
Anti CD28	T (Jurkat)	h	Verweij *et al.*, 1991
Anti-αβ TCR	T (SPB21)	h	Hazan *et al.*, 1990
Antigen	T (SPB21)	h	Hazan *et al.*, 1990
	T (A-E7)	m	Kang *et al.*, 1992a
c-AMP	Pre-B (70Z/3)	m	Shirakawa *et al.*, 1989
UV light	HeLa	h	Stein *et al.*, 1989a; b
4-Nitroquinoline oxide	HeLa	h	Stein *et al.*, 1989a
H_2O_2	T (Jurkat)	h	Schreck *et al.*, 1991
Theilera parva (parasite)	T cells	b	Ivanov *et al.*, 1989
Serum	BALB/c 3T3	m	Baldwin *et al.*, 1991
Okadaic acid	T (Jurkat)	h	Thevenin *et al.*, 1990
Viruses			
CMV	T (Jurkat) or HF (foreskin)	h	Sambucetti *et al.*, 1989
Adenovirus	Fibroblast (3T3)	m	Shurman *et al.*, 1989
	Cervical carcinoma (HeLa)	h	Nielsch *et al.*, 1991
Hepatitis B	Hepatocyte (HepG2)	h	Twu *et al.*, 1989a; Faktor and Shawl, 1990
Sendai	Fibroblast (L929)	m	Lenardo *et al.*, 1989
Herpes simplex, type I	T (Jurkat)	h	Gimble *et al.*, 1988
Human herpes virus, type VI	T (Molt3)	h	Ensoli *et al.*, 1989

indicated that phorbol esters gave maximal induction in 30 min and later studies showed significant induction occurred at much earlier times (Sen and Baltimore, 1986b; vide infra). Also, the kinetics were not identical for different inducers: phorbol stimulation of 70Z/3 cells remained maximal from 0.5 to 6 hr and was gone at 8 hr, whereas LPS induction became maximal at 2 hr and was still apparent at 8 hr (Sen and Baltimore, 1986b).

NF-κB induction of specific genes will be discussed in association with the physiologic role that they play.

A. T-Lymphocyte Activation

An important subset within the wide variety of genes controlled by NF-κB is the collection of genes that are induced during T-cell activation. Over 100 genes appear to be transcribed in T cells after stimulation by antigen in the context of an appropriate major histocompatability complex (MHC) molecule or agents that mimic this interaction (Zipfel *et al.*, 1989; Irving *et al.*, 1989; Crabtree, 1989). Therefore understanding gene control during T-cell activation should provide significant insights into the general molecular regulation of this process (Ullman *et al.*, 1990). NF-κB in cooperation with other transcriptional regulators (activators and repressors) appears to be involved in the expression of many genes especially during the early phases of the response to T-cell receptor stimulation (Jamieson *et al.*, 1991; Lenardo and Baltimore, 1989). Cytokine gene expression provides a good model for understanding NF-κB's role early in T-cell activation. mRNAs for cytokines, or more specifically interleukins, are usually undetectable in T cells unless the appropriate stimuli have been administered (DeMaeyer and DeMaeyer-Guignard, 1988). Though there has been some controversy regarding whether transcriptional or post-transcriptional mechanisms are the prevailing determinant in elevating mRNA levels, careful analysis of cytokine regulation in T cells indicates a major role for transcriptional control (Brorson *et al.*, 1991). The process initiates rapidly since increases in cytokine mRNAs are detected within 2 hr and in some cases peak at 4 hr. NF-κB is well suited for cytokine control because of the speed of its activation and its independence from new protein synthesis.

The best studied examples of T-cell cytokine gene regulation, IL-2 and the IL-2 receptor α chain genes, illustrate how NF-κB plays only one part in an ensemble of regulatory factors. A region of approximately 300 bp located immediately upstream of the transcription start site of the IL-2 gene, variously called the enhancer or the promoter, has been shown to strongly respond to T-cell activation signals (Brunvand *et al.*, 1988; Fujita *et al.*, 1986; Durand *et al.*, 1987; Novak *et al.*,1990). This DNA segment contains binding sites for NF-κB and other nuclear trans-activating proteins including the nuclear factor of activated T cells (NF-AT), octamer factors, AP-1, purine-rich binding factors, and, at least in human cells, a CD28-responsive complex (Lenardo *et al.*, 1988; Durand *et al.*, 1988; Hoyos *et al.*, 1989; Muegge *et al.*, 1989; Randak *et al.*, 1990; Fraser *et al.*, 1991; Granelli-Piperno and Nolan, 1991; Hentsch *et al.*, 1992). Deletion studies suggest that multiple protein binding sites must be occupied in order to allow full IL-2 enhancer activity (Durand *et al.*, 1988, Serfling *et al.*, 1989; Hoyos *et al.*, 1989). Certain of these are factors are tissue-specific, such as NF-AT; however, others such as AP-1 and NF-κB do not have any obvious tissue restriction but are important in regulatory states such as clonal anergy (Kang *et al.*, 1992b). The molecular switch that activates the IL-2 gene appears to be the constellation of trans-activators rather than any one taking a leading role.

Studies in which point mutations were introduced into the promoter clearly indicated a potentially important role for κB binding motif (IL-2-κB), presumably due to trans-activation by NF-κB or other Rel family members (Ballard *et al.*, 1988; Shibuya *et al.*, 1989; Shibuya and Taniguchi, 1989). Studies have also provided evidence that in certain cell lines the IL-2-κB site may function in a tissue-specific manner (Radler-Pohl *et al.*, 1990; Briegel *et al.*, 1991). Recently, evidence from the study of nontransformed murine T-cell clones indicates that more than one distinct *rel* family member acts at the IL-2-κB sequence (Kang *et al.*, 1992a). A complex, termed NF-κC, that comprises the p50 subunit of NF-κB and is distinct from the NF-κB p50/p65 heterodimer is present in unstimulated T cells but disappears after activation. Activation signals that lead to loss of NF-κC induce IL-2 mRNA. By contrast, conditions that elevate nuclear NF-κB or NF-AT levels but do not lead to loss of NF-κC fail to induce IL-2 mRNA. NF-κC removal appears to be due to its sequestration in the nucleus by a novel inhibitory protein, which we informally refer to as I-κC, whose synthesis is tightly linked to antigen stimulation and sensitive to cyclosporin A. One molecule that may serve an I-κC-like function is the Bcl-3 protein that is located in the nucleus and inhibits the p50 homodimer complex (Wulczyn *et al.*, 1992; G. P. Nolan and D. Baltimore, unpublished results). This pathway illustrates a mechanism by which NF-κB-related complexes can be regulated exclusively within the nucleus during activation of T cells. It also maintains tight regulation of the IL-2 gene, since NF-κB can be activated in response to a variety of signals but the loss of the NF-κC protein is directly linked to antigen stimulation (Kang *et al.*, 1992a). Recent results in human cells suggest that the mechanism described by Kang *et al.* may govern the transcription of HIV-1 and that Bcl-3 serves as an inhibitor of the NF-κC or p50 homodimer complex (Franzoso *et al.*, 1992). In contrast to antigen, stimulation of nontransformed T cells with anti-CD3ε does not stimulate transcription through the IL-2-κB site (Jain *et al.*, 1992). Interestingly, the induction of NF-κB seems to be only weakly blocked by cyclosporin A at physiological doses, but there may be other mitogen-induced complexes binding to κB sites that are sensitive to this immunosuppressant (Randak *et al.*, 1990; Schmidt *et al.*, 1990; Banerjee *et al.*, 1991; Emmel *et al.*, 1989; Granelli-Piperno *et* *al.*, 1990; Brini *et al.*, 1990). In tumor cells, induction of NF-κB and the transcription activity of κB-binding elements stimulated by PMA appear to be more resistant to effects of cyclosporin A than inducing agents that cause increases in intracellular Ca^{2+} such as ionomycin and lectins (U. Siebenlist, personal communication).

The IL-2 receptor α chain (IL-2R) gene also displays an interesting regulatory pattern centered on an NF-κB binding motif in its promoter. Elevated cell surface expression of the IL-2 receptor α chain is a critical step in generating high affinity receptors for IL-2 during the response of T lymphocytes to antigen (Waldmann, 1986). Several groups have shown that the p65/p50 heterodimer of NF-κB or a related complex transduces T-cell activation signals into increased activity of the IL-2R promoter (Leung and Nabel, 1988; Bohnlein *et al.*, 1988, 1989b; Lowenthal *et al.*, 1989a,b; Cross *et al.*, 1989; Lin *et al.*, 1990). Significantly, IL-2R promoter activation by the Tax trans-activator gene of HTLV-1 or TNF-α also works through NF-κB (Fig. 3) (Cross *et al.*, 1987, 1989; Siekevitz *et al.*, 1987a; Ruben *et al.*, 1988; Lowenthal *et al.*, 1989a). By contrast, phorbol ester inducibility of the IL-2R promoter is mediated by a distinct sequence and did not involve the NF-κB binding site (see below) (Ballard *et al.*, 1989; Lin *et al.*, 1990). The κB motif in the IL-2R gene only weakly binds NF-κB and manifests a poorer response to trans-activation than other κB motifs (Shibuya *et al.*, 1989). Recently it has been found that the activity of the κB motif in T cells is due to cooperation between NF-κB and the serum responsive factor (SRF) and other promoter factors such as Sp1 (Pomerantz *et al.*, 1989; Ballard *et al.*, 1989; Toledano *et al.*, 1990; Roman *et al.*, 1990). Serum responsive factor appears to be present at very high levels in both resting and activated T cells and binds adjacent to NF-κB in the promoter region (Kuang *et al.*, 1993). Constitutively higher levels of NF-κB are associated with deregulation of IL-2 and its receptor in certain T-lymphoma lines that depend on IL-2 for autocrine growth (Hemar *et al.*, 1991a). It is notable that a putative transcription regulatory molecule unrelated to NF-κB, but that binds to an IL-2R promoter sequence encompassing the NF-κB site, has been recently cloned (Adams *et al.*, 1991).

A variety of other cytokines that are produced by activated T cells also appear to involve NF-κB in their transcriptional control. These include IL-6, which stimulates B cells to proliferate, differentiate and produce Ig (Shimizu *et al.*, 1990; Libermann and Baltimore, 1990); GM-CSF, which sustains proliferation of bone marrow precursors for macrophages and granulocytes (Sugimoto *et al.*, 1990; Shreck *et al.*, 1990); TNF-α and -β, which are inflammatory mediators (Messer *et al.*, 1990; Paul *et al.*, 1990; Collart *et al.*, 1990; Goldfeld and Maniatis, 1989; Shakhov *et al.*, 1990; Hass *et al.*, 1990; Drouet *et al.*, 1991; Kuprash and Nedospasov, 1992;

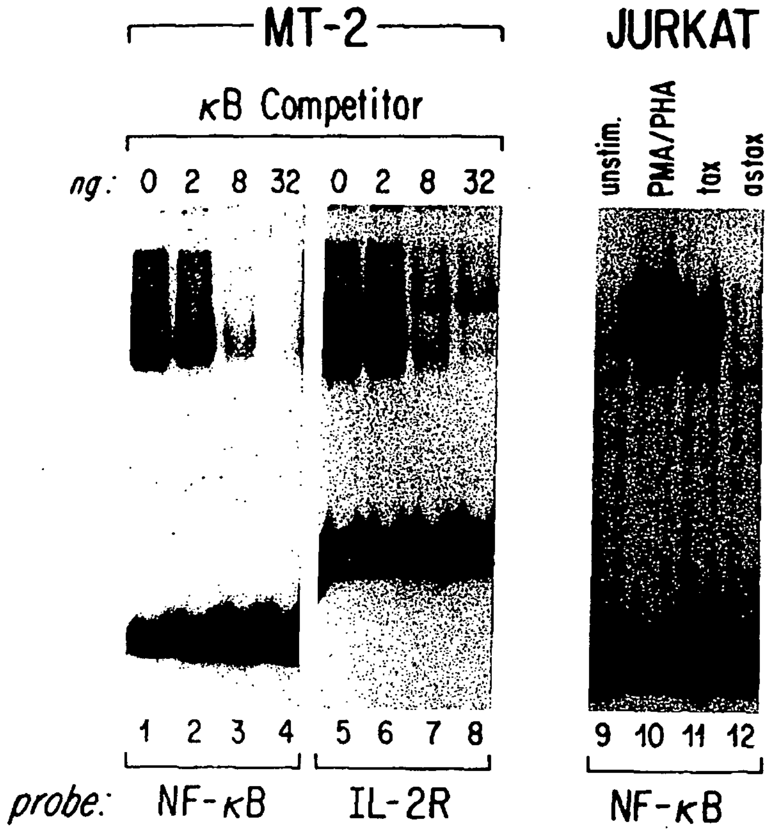


FIG. 3 Mobility shift electrophoresis analysis of nuclear extracts from the HTLV-1 infected human T-lymphoid tumor line MT-2 or the human T-lymphoma cell Jurkat. Lanes 1–4 show the complex detected with the κB binding site from the Ig κ gene, competed with increasing amounts of the same sequence to indicate specificity of the nucleoprotein complex. Lanes 5–8 show that a comigrating specific complex is formed with the κB binding site from the *IL-2 receptor (IL-2R) α chain* promoter. Lanes 9–12 are the complexes detected using the κB binding site from the Ig κ gene in nuclear extracts from Jurkat cells with unstimulated, stimulated with phorbol ester and phytohemagglutinin (PMA/PHA), or stably transfected with expression plasmids containing the HTLV-1 Tax coding sequence in the sense (tax) or antisense orientation (astax). These results have been previously described in Cross *et al.* (1989), and Leonardo *et al.* (1988).

Zuckerman and Evans, 1992); and proenkephalin, a precursor for neuropeptides whose mRNA is expressed, for unknown reasons, at high levels in activated T-helper lymphocytes (Rattner *et al.*, 1991). Interestingly, in the case of TNF-α and -β, these same cytokines are potent NF-κB inducers indicating that an autostimulatory loop could be set into motion in the response to antigen (Griffin *et al.*, 1989; Hohmann *et al.*, 1991a). Therefore NF-κB appears to form part of a coordinated program that underlies the immune response.

Precisely how NF-κB connects to signals initiated by the antigen receptor is still obscure. Different answers are obtained in various cellular models that have been studied. In tumor cells, often a relatively simple signal will suffice. For example, in EL-4 murine thymoma cells, PMA or lectin alone will induce NF-κB (Lacoste *et al.*, 1990; Shibuya and Taniguchi, 1989). PMA alone also induces NF-κB in non-T cells (Nelson *et al.*, 1988). In Jurkat both PMA and phytohemagglutinin together give an even greater induction than either agent alone (Hoyos *et al.*, 1989). The fact that induction of NF-κB by PMA and phytohemagglutinin is not blocked by physiologic concentrations of cyclosporin A in this system has been taken to indicate that NF-κB itself is not involved in the immunosuppression caused by this drug (Emmel *et al.*, 1989; Mattila *et al.*, 1990). In evaluating other lines, especially nontransformed T lymphocytes, PMA does not induce NF-κB (Kang *et al.*, 1991). Stimulation of the T-cell antigen receptor by physiologic ligands appears to activate NF-κB (Jamieson *et al.*, 1991; Kang *et al.*, 1992a). Signaling pathways through other T-cell surface markers such as CD28 also activates NF-κB (Gruters *et al.*, 1991; Verweij *et al.*, 1991; Granelli-Piperno and Nolan, 1991).

In many types of cells, including T cells, a single cytokine such as IL-1 or TNF-α will induce NF-κB (Krasnow *et al.*, 1991; Lacoste *et al.*, 1990; Hohmann *et al.*, 1990b). It is unclear, however, how many and what types of different signal transduction pathways are activated following engagement of the receptors for these cytokines. Some evidence suggests that TNF induction does not go through protein kinase C, at least in Jurkat T cells (Meichle *et al.*, 1990). Furthermore, the pathways activated by an individual cytokine may differ between cell types: TNF induction of NF-κB is increased by cycloheximide in Jurkat cells, but blocked by cycloheximide in HepG2 cells (Banerjee *et al.*, 1989). As in the case of NF-κB induction in B cells, other signal transduction pathways in T cells, especially tyrosine phosphorylation, have not been evaluated.

One interesting observation is that a decrease in the level of intracellular thiols can be one of the steps in NF-κB induction (Staal *et al.*, 1990). For example, the induction of NF-κB by TNF-α or phorbol ester can be blocked by *N*-acetylcysteine, an agent that will maintain high levels of glutathione and other thiol compounds. It has been recently reported that this may be a useful *intervention to prevent the activation of HIV* transcription (Roederer *et al.*, 1990; Kalebic *et al.*, 1991). Reducing agents have the capacity to release NF-κB from I-κB in cytosolic extracts (Toledano and Leonard, 1991). It has also been found that H_2O_2 can induce NF-κB and the replication of a latent HIV provirus in a human T lymphoma. This presumably occurs by generating reactive oxygen intermediates, and can be blocked with *N*-acetylcysteine, dithiocarbamates, and metal chelators (Schreck *et al.*, 1991, 1992).

B. Other Genes Involved in Immune or Inflammatory Function

Certain cytokines that are primarily derived from leukocytes that are not lymphocytes are also controlled by NF-κB. These include G-CSF, a growth cytokine for granulocytes (Nishizawa *et al.*, 1990; Nishizawa and Nagata, 1990); TNF-α and IL-6, made primarily by monocytes but also by T cells (Collart *et al.*, 1990; Shakhov *et al.*, 1990; Libermann and Baltimore, 1990; Zhang *et al.*, 1990; Shimizu *et al.*, 1990); and IL-8, a chemotactic factor that recruits neutrophils into inflammatory sites (Mukaida *et al.*, 1990). Recent evidence has shown that NF-κB physically interacts with the NF-IL-6 transcription factor which is an important regulator of the IL-6 and IL-8 genes (K. LeClair, *personal communication*). The involvement of NF-κB in β-interferon (β-IFN) gene induction was first reported by Visvanathan and Goodbourne (1989). They found that double-stranded RNA induced a binding complex with characteristics of NF-κB and pointed out the possibility that multiple kinases, including DI kinase, may release NF-κB from I-κB. Similar work by others showed that virus infection can also induce NF-κB (Lenardo *et al.*, 1989; Fujita *et al.*, 1989b; Hiscott *et al.*, 1989). NF-κB binds to and positively activates a site called PRD-II in the β-interferon promoter. As such it is part of a group of virus-regulated events including activation by two positive effectors and the release of a repressor. Because the β-interferon gene can be induced in almost every cell type, NF-κB is likely involved in gene regulation in perhaps all nucleated cells. Curiously, another factor that binds to the PRD-I motif of the β-interferon promoter, IRF-1, is itself regulated by NF-κB, but its role in β-interferon activation is controversial (Fujita *et al.*, 1989a; Baeuerle, 1991; Pine *et al.*, 1990; Reis *et al.*, 1992). Other investigators have provided evidence that the distinct expression of α- and β-interferons is due to different virally induced transcription factors since the α1-interferon promoter is not regulated by NF-κB (MacDonald *et al.*, 1990).

Key tasks of immune recognition may be carried out by NF-κB. For example, the ability of specialized cells such as B cells, macrophages, and dendritic cells to participate in antigen activation of T cells seems to involve gene control by NF-κB. This regulation embraces the genes for molecules, mostly receptors, that play key roles in the events of antigen processing, presentation, or recognition: MHC class I and II; β_2-microglobulin molecules; MHC class II molecules: the invariant chain, Ii; and the T-cell antigen receptor β chain (Baldwin and Sharp, 1988; Israel *et al.*, 1987; Jamieson *et al.*, 1989; Blanar *et al.*, 1989; Mauxion *et al.*, 1990; Schwemmle *et al.*, 1991; Pessara and Koch, 1990; Blanchet *et al.*, 1992).

Another inflammatory response that involves NF-κB and gene activation is the acute phase response of hepatocytes (Edbrooke *et al.*, 1989, 1991). During this process, various cytokines (IL-1, IL-6, and TNF-α) acting at the liver cell membrane and steroid hormones acting at the nucleus produce an increase in the expression of particular genes and NF-κB is the mediator for elevated expression of the gene for serum amyloids A2β and A1 (Edbrooke *et al.*, 1989; Li and Liao, 1991). SAA2β is the precursor of amyloid A protein subunits of amyloid fibrils in secondary amyloidosis (Edbrooke *et al.*, 1989). Other acute phase genes—angiotensinogen, a precursor for the potent pressor hormone angiotensin II, and α_1-acid glycoprotein, a nonspecific immunosuppressant—are also controlled by NF-κB or a trans-activator of related specificity (Ron *et al.*, 1990a,b,c; Brasier *et al.*, 1990; Won and Baumann, 1990).

Other hepatocyte genes loosely included under the rubric of immune/inflammatory genes which are apparently regulated by NF-κB include the complement factor 4 and factor B genes (Yu *et al.*, 1989; Nonaka and Huang, 1990). The factor B protease is a member of the complement cascade that plays a critical role in the activation of the alternative pathway and its induction by TNF-α seems due to NF-κB (Nonaka and Huang, 1990).

Data supporting a role for NF-κB in the direct regulation of cell growth are emerging. Renan (1989) and Duyao *et al.* (1990) have both suggested that NF-κB takes part in a novel type of growth gene regulation. By binding to a site in the first exon of the c-*myc* mRNA, NF-κB could act to relieve transcriptional blockade which governs the level of full-length mRNA produced. NF-κB would interrupt a stable stem–loop structure in this region of the c-*myc* message that appears to halt transcriptional elongation by RNA polymerase (Duyao *et al.*, 1990). Another set of growth-related genes, the *gro* family, which are transiently induced by serum stimulation and may participate in the growth of melanomas, involve NF-κB when actuated by IL-1 and TNF-α (Anisowicz *et al.*, 1991). Lastly, Baldwin and colleagues have found that serum stimulation of BALB/c 3T3 cells leads to the presence of a binding complex resembling NF-κB (1991). Collectively, these findings support the notion that NF-κB may be a central regulator of genes involved in cell growth in both lymphocytes and nonlymphoid cells.

Other genes whose expression is influenced by NF-κB or its congeners cannot be characterized as strictly immunological or inflammatory. These diverse targets for NF-κB complete the picture of a very versatile transcription factor. NF-κB or a member of the family of zinc-finger proteins that bind to κB-related motifs may regulate a site in the gene for the αI-crystallin protein that is a constituent of the lens of the eye (Nakamura *et al.*, 1990). The promoter of the urokinase-type plasminogen activator has at least one or possibly multiple κB-like sites that bind complexes of the c-*rel* protein and p65 (Novak *et al.*, 1991; Hansen *et al.*, 1992). Also, NF-κB binding sites have been postulated by sequence homologies within a novel transcription unit in the rat insulin-like growth factor gene II (Matsuguchi *et al.*, 1989), the mouse perforin gene (Youn *et al.*, 1991), and the mouse MHC class II Eβ gene (Shenkar *et al.*, 1991). Finally many viral promoters and enhancers contain functional NF-κB sites which will be discussed further below.

V. Structure of NF-κB Sites: Clues for Specificity?

One of the features of NF-κB binding motifs is that they can differ quite significantly in their primary nucleotide sequence compared to the Ig-κB sequence (originally defined as the 10-nt 5′-GGGACTTTCC-3′). These variant sites can differ in their affinity for NF-κB and may be functionally distinct depending on the structure of the nucleoprotein complex formed (Lenardo and Baltimore, 1989; Baeuerle, 1991; Zabel *et al.*, 1991; Kunsch *et al.*, 1992). These sites have been summarized in Table II. At a glance, it can be noticed that most NF-κB motifs are asymmetric. The first three nucleotides are invariably guanine and the last two are virtually always cytosine. As one moves from 5′ to 3′ through the nucleotide sequence, the complexion of the motif shifts from primarily purines to pyrimidines. The nucleotides in the very middle are quite variable (but they tend not to be G in the alignments depicted) and may contribute very little to binding. For example, in comparing the NF-κB motifs from the Ig κ genes in different species, they appear as versions of the mouse site in which either one (rabbit) or both (human) of the central nucleotides have been lost (see Table II) (Emorine *et al.*, 1983). Nonetheless, the central nucleotides may play a role in the ultimate structure of the nucleoprotein complex (Schreck *et al.*, 1990).

TABLE II
NF-κB Target Genes

	Gene	Sequence[a]	Reference
Well defined a. Surface molecules involved in immune function	Ig κ light chain (mouse)	GGGACTTTCC	Sen and Baltimore, 1986a
	Ig κ light chain (human)	GGGGATTTCC	Emorine *et al.*, 1983
	Ig κ light chain (rabbit)	GGGGTTTCCC	
	IL-2Rα chain	GGGAATCTCC	Ballard *et al.*, 1988 Leung and Nabel, 1988
	TCR β chain	GGGAGATTCC	Jamieson *et al.*, 1989
	MHC class I (H-2K^b)	GGGGATTCCC GGGGAAGCCC	Baldwin and Sharp, 1988 Israel *et al.*, 1989
	MHC class II (Eα^d) (Eβ)	GGGACTTCCC	Blanar *et al.*, 1989
	Invariant chain Ii	GGGAATTTCC	Pessara and Koch, 1990
	β2 microglobulin	GGGACTTTCC	Israel *et al.*, 1989
	ELAM-1	GGGGATTTCC	Whelan *et al.*, 1991
b. Soluble molecules involved in immune function	IL-2	GGGATTTCAC	Lenardo *et al.*, 1988
	IL-6	GGGATTTTCC	Shimizu *et al.*, 1990 Lieberman and Baltimore, 1990
	IL-8 (human)	GGAATTTCCT	Mukaida *et al.*, 1990 Mahe *et al.*, 1991
	GM-CSF (mouse)	GGGAACTACC	Sugimoto *et al.*, 1990 Schreck *et al.*, 1990
	G-CSF	GGGGAATCTC GGG(AA)AT(CAC)	Nishizawa *et al.*, 1990
	TNF-α (cachectin)	GGGGCTTTCC GGGAATTCAC GGGGAATCCT GGGGCTGCCC GGGAATTTCC	Shakhov *et al.*, 1990 Collart *et al.*, 1990 Kuprash and Nedospasov, 1992
	TNF-β (lymphotoxin)	GGGGCTTCCC	Paul *et al.*, 1990 Messer *et al.*, 1990
	β-interferon	GGGAAATTCC	Visvanathan and Goodbourn, 1989 Fujita *et al.*, 1989b Lenardo *et al.*, 1989 Hiscott *et al.*, 1989
	Proenkephalin (rat)	GGGACGTCCC	Rattner *et al.*, 1991
c. Acute phase response genes	Serum amyloid A (SAA)	GGGACTTTCC	Edbrooke *et al.*, 1989
	Angiotensinogen (rat)	GGGATTTCCC	Ron *et al.*, 1990a
	Complement factor B (mouse)	GGGAATCCCC	Nonaka and Huang, 1990 Visvanathan and Goodbourn, 1989
	Complement C4 protein	GGGAAGTCCC	Yu *et al.*, 1989
d. Oncogenes	c-*myc*	GGGAAAACCC	Duyao *et al.*, 1990
e. Viruses	HIV	GGGACTTTCC	Nabel and Baltimore, 1987
	SV-40	GGGACTTTCC GGGAAGTACC	Kanno *et al.*, 1989 Macchi *et al.*, 1989
	Cytomegalovirus	GGGACTTTCC GGGGATTTCC	Sambucetti *et al.*, 1989
	Adenovirus	GGGACTTTCC	Williams *et al.*, 1990
f. Others	Invariant chain	GGGAATTTCC	Pessara and Koch, 1990
	Vimentin	GGGGCTTTCC	Lilienbaum *et al.*, 1990
	Gro		Anisowicz *et al.*, 1991
	Interferon regulatory factor	GGGGAATCCC	Baeuerle, 1991
	Consensus:	GGGRNNYYCC	

Nucleotide distribution

Position:	1	2	3	4	5	6	7	8	9	10	
	40	40	40	13	1	3	2	0	0	0	G
	0	0	1	27	21	8	1	2	3	0	A
	0	0	0	0	5	28	35	20	1	2	T
	0	0	0	0	13	1	2	18	36	38	C

	Gene	Sequence[a]	Reference
Undefined (genes containing potential NF-κB sites)			
	Perforin	GGGCCTTTCA	Youn *et al.*, 1991
	Insulin-like growth factor II	TGGGGAGCCCCC AGGGGCTCCCCAG	Matsuguchi *et al.*, 1989
	1731 LTR (retrotransposon)	AGGCAATTTCCA	Ziarczyk *et al.*, 1989
	TGF β2	GGGCATTGAC	Malipiero *et al.*, 1990
	Apolipoprotein CIII	GGGAAATCCC	Reue *et al.*, 1988
	Urokinase		Novak *et al.*, 1991; Hansen *et al.*, 1992
	ICAM-1	GGAGGATTCC GGGGAAGTCC	Voraberger *et al.*, 1991 Stade *et al.*, 1990
	CD7	GGGAGTCCCT GGGACGCCCT	Schanberg *et al.*, 1991

[a] Note that additional variant 10-bp binding sequences can be obtained by shifting the site one or two nucleotides upstream or downstream or by the sequence encoded by the opposite strand in many genes.

As will be recounted in more detail below, NF-κB comprises two subunits, p50 and p65, which can bind individually as homodimers or as a p50/p65 heterodimer. A careful comparison of the binding of either p50 or p65 homodimers to the p50/p65 heterodimer indicates that p50 apparently binds to the 5′ (more conserved) half site and p65 binds to the 3′ (less conserved) half site (Urban *et al.*, 1991). Does this mean that different versions of the κB motif prefer p50 in combination with different partners from the Rel family? Direct evidence is just emerging that this will likely be an important theme in the future (Perkins *et al.*, 1992). As might be expected, the binding of homodimers of Rel proteins is strongest to sites that are palindromic or otherwise highly symmetric (Urban *et al.*, 1991). In any case, it is clear that variations in the nucleotide sequence of the κB motif have an important influence on function.

How could specificity be encoded in the various versions of the κB motif? One obvious way is simple affinity—if a site was of high affinity, it would respond to lower levels of NF-κB, whereas a weaker site would be relatively insensitive to NF-κB. This type of functional distinction has been pointed out (Cross *et al.*, 1989; Kuang *et al.*, 1992). In fact, weaker binding may lead to a greater dependence of the κB motif on other factors within a given control element (Kuang *et al.*, 1992). A second means to achieve specificity would be the ability of a given site to discriminate between different members of the κB binding protein family (Mauxion and Sen, 1989; Perkins *et al.*, 1992). A third mechanism could be an alteration of the structure of the DNA binding site caused by factor binding. Evidence has been obtained that bending of the κB DNA sequence is induced by interactions with various Rel proteins (Schreck *et al.*, 1990; Kuprash and Nedospasov, 1992). Moreover, the angle of the bent site differs depending on whether NF-κB or other *rel* complexes are bound (Schreck *et al.*, 1990; Kuprash and Nedospasov, 1992). Fourth, the sequences surrounding an NF-κB site could have effects on both binding and function of NF-κB.

Finally, a more subtle and perhaps more powerful mechanism would involve the DNA site acting as a ligand that causes an allosteric change in the Rel protein complex. Depending on the primary sequence of the κB binding motif, it could be envisioned that the bound factor could adopt different structures that have different functional consequences for transcription. Evidence supporting such a mechanism has been recently obtained in studies using purified NF-κB subunits in transcription and binding assays *in vitro* (Fujita *et al.*, 1992). They find that p50 homodimers activate transcription through the κB site from the MHC class I gene but not that from the β-interferon gene even though binding affinity for both is equivalent. The NF-κB p50/p65 heterodimer did not show differential activation of the two sites. This type of "induced fit" may also be determined to some extent by structural variations for *rel* proteins in solution: p50 appears to adopt a globular shape in solution though p65 or p50/p65 heterodimers seem to be more elongated (Schreck *et al.*, 1990).

VI. The Biochemistry of NF-κB

The cardinal feature of NF-κB regulation is that at least two different cellular forms of NF-κB exist: (*a*) a form that is competent to bind DNA that is constitutively present in mature B lymphocytes and some T lymphocytes and monocytes (Sen and Baltimore, 1986a; Cross *et al.*, 1989; Griffin *et al.*, 1989) and (*b*) a non-DNA-binding form that is present in the cytoplasm of many, or perhaps all, cell types. The cytoplasmic form can be induced to translocate to the nucleus and bind DNA following a great variety of cellular stimuli (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a). A key question, therefore, was how these forms of NF-κB differ biochemically. Considerable light was shed on this problem by the description of a highly specific and reversible protein inhibitor of NF-κB (Baeuerle and Baltimore, 1988a, b; Baeuerle *et al.*, 1988). This was first revealed by using dissociating treatments such as formamide or sodium deoxycholate to separate NF-κB from its inhibitor, I-κB, in cytosolic extracts (Baeuerle and Baltimore, 1988a; Harel-Bellan *et al.*, 1989). I-κB appeared to be noncovalently associated with NF-κB. The presence of a metastable protein inhibitor of NF-κB fits well with the fact that protein synthesis inhibitors cause superinduction of NF-κB (presumably by preventing new synthesis of the inhibitor) (Sen and Baltimore, 1986b). Also, the finding that phorbol ester induction reduced the amount of cytosolic NF-κB and increased the nuclear form gave rise to the idea that preexisting NF-κB in the cytoplasm was freed from an inhibitor and shuttled into the nucleus (Baeuerle and Baltimore, 1988b). Because NF-κB and I-κB do not freely exist in the cytosolic extracts, it is likely that each is present in stoichiometrical amounts (Baeuerle and Baltimore, 1988b). Though the true range of regulatory interactions is much more complex than this simple formulation, it represents a central theme in the inducible control of NF-κB.

Original purifications of NF-κB indicated that the binding activity was contained in proteins in the 40,000–60,000 molecular weight (MW) range (Kawakami *et al.*, 1988; Lenardo *et al.*, 1988). These analyses also showed that NF-κB was a positive regulator of transcription *in vitro* (Kawakami *et al.*, 1988) and revealed the interesting property that NF-κB binding could be dramatically stimulated by the addition of nucleoside triphosphates (Lenardo *et al.*, 1988). Other studies showed that several agents will increase binding including spermine, spermidine, and other multivalent cations (Schreck *et al.*, 1990). Subsequent biochemical analysis revealed that the NF-κB mobility shift complex comprised two distinct subunits. These components of 50,000 and 65,000 MW have been referred to as p50 and p65, respectively (Baeuerle and Baltimore, 1989). Initially, it was proposed that p50 but not p65 was a DNA binding subunit and that together they formed a heterotetrameric complex in which two p50 subunits contacted each half of the NF-κB binding site (Baeuerle and Baltimore, 1989). However, subsequent studies have determined that p65 does interact with DNA and, in fact, may dictate the binding strength of the p50/p65 heterodimer to NF-κB motifs of certain sequence (see below; Nolan *et al.*, 1991; Urban *et al.*, 1991). Importantly, p65 and p50 each interact with different forms of I-κB introducing an important mode of regulation that has the capacity to distinguish κB site binding complexes of divergent subunit compositions (Kerr *et al.*, 1991; Inoue *et al.*, 1992a).

The p50/p65 complex obtained from the nuclei of stimulated cells was biochemically indistinguishable from that obtained from the cytosol of unstimulated cells (Baeuerle and Baltimore, 1988c; Baeuerle *et al.*, 1988).

Complexes from either source could be inhibited by partially purified I-κB. Thus, the induction process appeared to result from a change in I-κB rather than NF-κB. Evidence for biochemical modulation of I-κB came from studies in which treatment of cytosolic extracts with a variety of kinases including protein kinase A or C (PKA or PKC), or heme-regulated eIF-2 kinase (HRI) released NF-κB from its inhibition by I-κB (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Moreover, whereas purified and unmodified I-κB had potent NF-κB inhibitory activity, I-κB that had been phosphorylated by either PKC or HRI kinases lost all of its NF-κB inhibitory activity (Ghosh and Baltimore, 1990). However, PKA did not phosphorylate or abrogate the inhibitory activity of purified I-κB. This suggested that PKA might release NF-κB from a complex with I-κB through an indirect mechanism (Ghosh and Baltimore, 1990). Interestingly, a 65-kDa protein that binds to κB sites has been shown to be physically associated with a serine/threonine kinase (Ostrowski *et al.*, 1991). In summary, there appears to be no single kinase or underlying mechanism that governs all NF-κB induction events (see below). For example, though PMA induces NF-κB in many cell types, NF-κB induction by IL-1 or LPS seems not to be mediated by PKC (Bomsztyk *et al.*, 1991). Also, dephosphorylation of I-κB may also accentuate its release from NF-κB (Hohmann *et al.*, 1992; Link *et al.*, 1992). Nonetheless, the release of NF-κB via I-κB phosphorylation provides an elegant explanation for the missing link between NF-κB regulation of nuclear genes and signal transduction pathways from the cell surface.

Biochemical analysis of I-κB purified from cytosolic extracts of human placenta revealed that it exists in two isoforms that can be separated chromatographically: I-κBα, a 35- to 37-kDa polypeptide (Ghosh and Baltimore, 1990; Zabel and Baeuerle, 1990), and a minor form (~20–30% of the placental inhibitory activity), I-κBβ, a 43-kDa polypeptide (Zabel and Baeuerle, 1990). Interestingly, an early report of an I-κB activity from the cytosol of unstimulated 70Z/3 cells that had a molecular size of ~60 kDa by gel filtration was likely a distinct form of I-κB (I-κBγ) that was recently found to be specific for lymphoid cells (Baeuerle and Baltimore, 1988b; Inoue *et al.*, 1992a). I-κBα and I-κBβ appear to interact with p65 but not the p50 subunit. Other studies showed that I-κBβ also bound to and inhibited c-*rel* DNA binding (Kerr *et al.*, 1991). Importantly, p50 heterodimers with either p65 as well as c-*rel* and p65 or c-*rel* homodimers, but not p50 homodimers, are targets for inhibition (Urban and Baeuerle, 1990; Kerr *et al.*, 1991; G. Nolan and D. Baltimore, personal communication). Therefore p65 or Rel appear to be "receptors" for I-κBα and I-κBβ. By contrast, I-κBγ interacts with p50 homodimers, p50/p65 heterodimers, or Rel (Inoue *et al.*, 1992a). The presence or absence of various I-κBs may determine the expression of complexes that comprise only p50 or p50 paired with other *rel* proteins.

Though both the α and the β isoforms of I-κB are modulated by phosphorylation, interesting differences have been found. Apparently the inhibitory activity of I-κBβ can be abrogated by phosphorylation with PKA and PKC, whereas I-κBα is only affected by PKC (Kerr *et al.*, 1991). Furthermore, phosphatase treatment is able to extinguish the NF-κB inhibitory activity of I-κBβ but does not affect I-κBα (Baeuerle, 1991). *In vitro* experiments have shown that either isoform of I-κB can dissociate a preformed complex between NF-κB and its DNA binding site (Zabel and Baeuerle, 1990). It is possible that I-κB could actively reverse NF-κB-mediated gene regulation, but it is not clear whether I-κB molecules have nuclear localization signals that would allow them to reach NF-κB bound in the chromatin (Zabel and Baeuerle, 1990; Haskill *et al.*, 1991). Further experiments will be required to elucidate the various cellular roles of I-κB isoforms. Possible mechanisms for controlling the nuclear transport of NF-κB and other gene regulatory proteins have been recently discussed (Silver, 1991; Schmitz *et al.*, 1991).

VII. Molecular Cloning of NF-κB and I-κB

The recent molecular cloning of cDNAs encoding p50, p65, and I-κB-like molecules has substantially enhanced our overall understanding of this transcription regulatory system (Ghosh *et al.*,1990; Kieran *et al.*, 1990; Bours *et al.*, 1990, 1992; Nolan *et al.*, 1991; Meyer *et al.*, 1991; Ruben *et al.*, 1991; Haskill *et al.*, 1991; Davis *et al.*, 1991; Inoue *et al.*, 1992b). Most significantly, the binding subunits of NF-κB are members of a family of proteins that are related to the *rel* oncogene (for an overview see Gilmore, 1990) (Figs. 4 and 5). These genes are related to the *Drosophila dorsal* gene product as well as a recently identified *Xenopus* gene, demonstrating that the Rel DNA binding protein structure arose early in evolution (Steward, 1987; Kao and Hopwood, 1991). Molecular cloning studies have now identified a number of Rel family members including p49 or p50B, p50, p65, *rel*B, and c-*rel* (Schmid *et al.*, 1991; Ghosh *et al.*, 1990; Nolan *et al.*, 1991; Kieran *et al.*, 1990; Meyer *et al.*, 1991; Bours *et al.*, 1990, 1992; Ryseck *et al.*, 1992; Wilhelmsen *et al.*, 1984). Significantly, proteins that have an I-κB-like inhibitory activity for NF-κB share a structural domain known as the "cell cycle" or "ankyrin" repeat. The binding subunits of the Rel family can form both homodimeric and heterodimeric complexes that differ in both their binding and their transcriptional properties. Heterodimeric regulatory systems allow for a greater combinatorial array of distinct gene regulatory molecules (Murre *et al.*, 1989; Bohmann *et al.*, 1988). Also, the Rel protein sequence does not match previously identified DNA binding or dimerization structures. Thus, the protein struc-

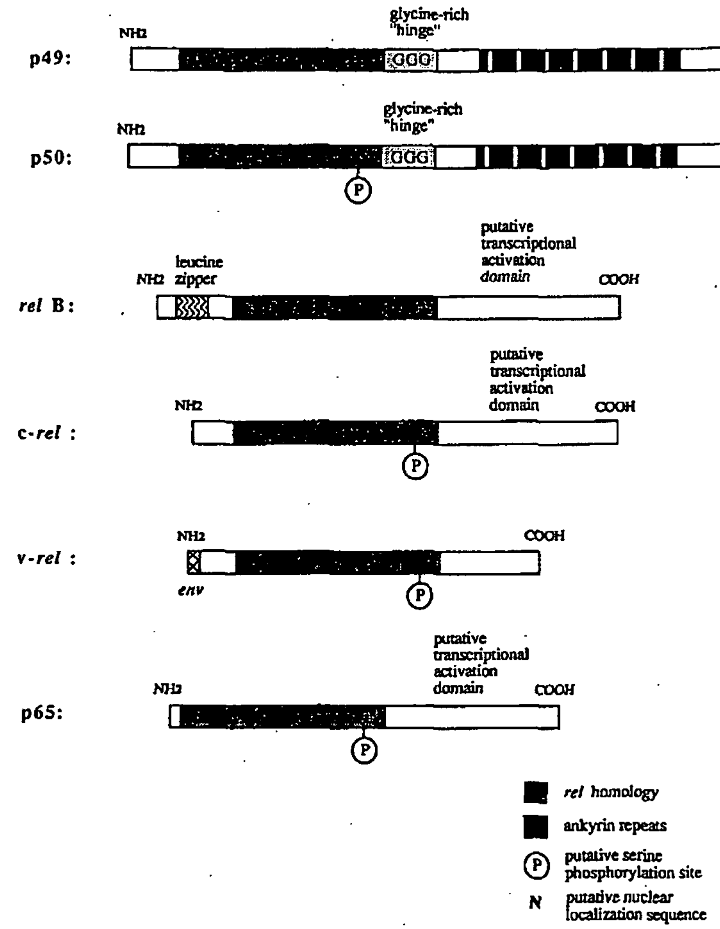

p49:
NH2
glycine-rich "hinge"
GGG
COOH
p50:
NH2
glycine-rich "hinge"
GGG
COOH
P
rel B:
NH2
leucine zipper
putative transcriptional activation domain
COOH
c-rel:
NH2
putative transcriptional activation domain
COOH
v-rel:
NH2
env
COOH
p65:
NH2
putative transcriptional activation domain
COOH
rel homology
ankyrin repeats
putative serine phosphorylation site
N putative nuclear localization sequence

FIG. 4 Shown schematically are the primary structures of the molecularly defined members of the Rel gene family. Only two (p50 and p49/p50B) have bipartite structures that include Rel and ankyrin repeat domains.

ture that governs the interactions among Rel subunits, the ankyrin repeat proteins, and DNA will be of great interest. Finally, it is notable that a chromosomal translocation juxtaposing the gene encoding p49/p50B, called lyt-10, with the immunoglobulin Cα-1 locus has been described in a patient with B-cell lymphoma (Neri *et al.*, 1991). The fusion gene created by the translocation encodes a *rel* DNA binding domain lacking the ankyrin repeat domain (see below), suggesting that the protein may be inappropriately regulated and play a role in oncogenesis. Chromosomal localization of the p49-p50B/p97 gene to 10q24 and p50/105 gene to 4q21.1-q24 by other workers places each of these genes at genomic positions associated with acute lymphatic leukemia (Liptay *et al.*, 1992; Ten *et al.*, 1992). Also, the c-*rel* gene has been mapped to the chromosomal region 2p12.2-13, which is rearranged in some large cell lymphoma (Brownell *et al.*, 1988; Lu *et al.*, 1991).

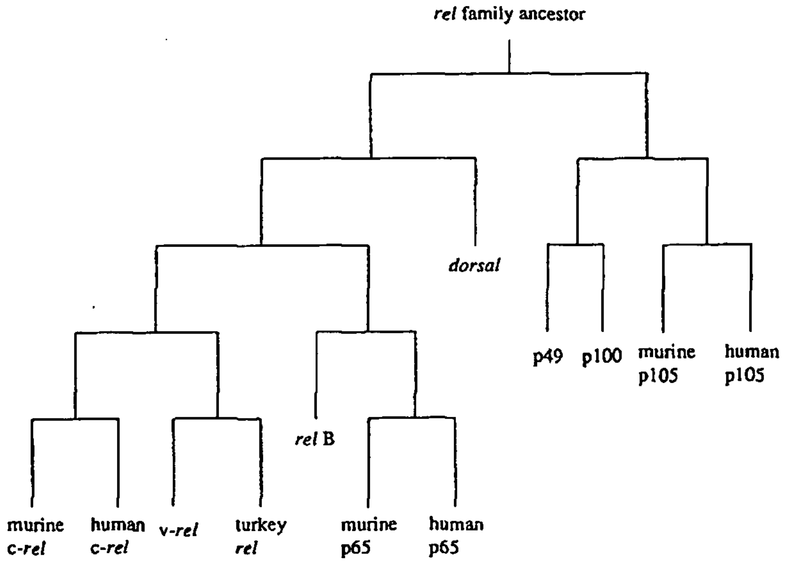


FIG. 5 Phylogenetic relationships between members of the Rel gene family in different species. This figure was adapted from Schmid *et al.* (1991).

A. Rel: The Cellular Counterpart of a Viral Oncogene

Determination of the primary sequence of the p50 cDNA first established the connection between NF-κB, the proteins encoded by the v-*rel* oncogene, its cellular counterpart c-*rel*, and the *Drosophila* maternal effect gene *dorsal* (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990; Meyer *et al.*, 1991). v-*rel* is the oncogene carried by Rev-T, a highly transforming defective avian retrovirus that causes a rapid and fatal lymphomatosis in young birds (Sylla and Temin, 1986; Rice and Gilden, 1988).

The viral genome encodes a single protein product, a 59-kDa phosphoprotein called p59$^{v\text{-}rel}$ (Gilmore and Temin, 1986; Herzog and Bose, 1986; Rice *et al.*, 1986; Walro *et al.*, 1987; Garson and Kang, 1990; Garson *et al.*, 1990). This protein is located mostly in the cytoplasm but also is present in small amounts (10–15%) in the nucleus.

Early studies suggested that *rel* proteins were involved in transcriptional control (Gelinas and Temin, 1988; Hannink and Temin, 1989; Bull *et al.*, 1990; Kamens *et al.*, 1990). Recent work shows that p59$^{v\text{-}rel}$ encodes a protein that binds to κB sites but appears to be incapable of stimulating transcription from reporter genes that depend on transactivation through κB sites (Inoue *et al.*, 1991; Ballard *et al.*, 1990; Kabrun *et al.*, 1991; Richardson and Gilmore, 1991). By contrast, the protein encoded by the c-*rel* oncogene, p85$^{c\text{-}rel}$, also binds with high affinity to κB sites but works as a transcriptional activator (Inoue *et al.*, 1991). In fact, v-*rel*, but not nontransforming mutants of v-*rel*, blocked transcriptional trans-activation by NF-κB (Ballard *et al.*, 1990). This effect could be due to either competition of the two factors at the level of DNA-binding or a "trans-dominant" effect in which p59$^{v\text{-}rel}$ inhibited NF-κB by a protein–protein interaction that sequesters one of its subunits (Ballard *et al.*, 1990; Inoue *et al.*, 1991). The mechanism of malignant transformation by Rel has been recently discussed more completely elsewhere (Gilmore, 1991).

v-*rel* and its cellular counterpart of c-*rel* have been cloned in birds (Chen *et al.*, 1983; Wilhelmsen *et al.*, 1984; Hannick and Temin, 1989) and in mammals (Grumont and Gerondakis, 1989, 1990a,b; Brownell *et al.*, 1985) (Fig. 5). For c-*rel*, the murine cDNA (Bull *et al.*, 1990), the human cDNA (Brownell *et al.*, 1989), and the chicken cDNA (Capobianco *et al.*, 1990) have all been cloned (Fig. 5). The avian tissues that have the highest c-*rel* expression contain the population of target cells for Rev-T. Indeed, although constitutive expression of c-*rel* is seen in many cell types, high levels are observed only in lymphoid cells (Herzog and Bose, 1986; Brownell *et al.*, 1987, 1988; Moore and Bose, 1989; Grumont and Gerondakis, 1990a,b; Zhang *et al.*, 1991). Expression of the c-*rel* gene is induced by serum or phorbol ester in fibroblasts or by phorbol ester and concanavalin A in T cells—conditions very similar to those that induce the mRNA for the p50 and p65 NF-κB subunits (see below; Grumont and Gerondakis, 1990a,b; Bull *et al.*, 1989). Recent characterization of the c-*rel* promoter has shown that it contains a consensus NF-κB site and that it can be directly repressed by v-*rel* but not other viral oncoproteins (Hannink and Temin, 1990; Capobianco and Gilmore, 1991). Interestingly, repression of the c-*rel* promoter can still take place even if the κB site is mutated (Capobianco and Gilmore, 1991).

Cloning of c-*rel* has revealed that v-*rel* encodes a truncated version of the c-*rel* protein product, p85$^{c\text{-}rel}$ (Stephens *et al.*, 1983; Wilhelmsen *et al.*, 1984; Hannink and Temin, 1989; Capobianco *et al.*, 1990, 1991; Grumont and Gerondakis, 1989; Brownell *et al.*, 1989). p59$^{v\text{-}rel}$ replaces two N-terminal amino acids of c-*rel* with a stretch of amino acids derived from the viral *env* gene. It also lacks 118 carboxyl-terminal amino acids that may include the transcription activating domain for p85$^{c\text{-}rel}$ (Bull *et al.*, 1990; Inoue *et al.*, 1991). The *rel* homology region appears to be critical for transformation (Kamens *et al.*, 1990; Garson and Kang, 1990). It is further notable that a protein kinase A site in the v-*rel* coding sequence also appears to be critical for both the transforming and the transcriptional repression functions of the p59$^{v\text{-}rel}$ protein (Mosialos *et al.*, 1991).

Intriguingly, p59$^{v\text{-}rel}$ isolated from cellular extracts is associated with at least five different proteins in a complex that has an apparent molecular mass of over 400 kDa (Lim-Tung *et al.*, 1988, 1990; Simek and Rice, 1989; Morrison *et al.*, 1989; Davis *et al.*, 1990; Kochel *et al.*, 1991). This is true whether p59$^{v\text{-}rel}$ is isolated from transformed B-cell lines, or overexpressed in other avian cell types (Kochel *et al.*, 1991). In view of the regulatory features known for complexes between NF-κB and I-κB, the so-called *rel*-associated proteins take on potential importance for understanding the biochemical mechanism of *rel* transformation. In the cytosol of transformed cells, p59$^{v\text{-}rel}$ is mainly associated with a 40-kDa phosphoprotein termed pp40; in smaller amounts with the constitutive avian heat shock protein hsp70; and with three other proteins, p75, p115, and p124; the nuclear p59 associates with pp40 (Davis *et al.*, 1990). pp40 appears to be the β form of I-κB and will be discussed below (Kerr *et al.*, 1991; R. Taub, personal communication). Strikingly, p75 is the product of the protooncogene c-*rel* (Simek and Rice, 1988). Moreover, p85$^{c\text{-}rel}$ has been found complexed with the very same proteins as v-*rel* especially the ~40-kDa and the 115-kDa proteins (Morrison *et al.*, 1989; Davis *et al.*, 1990; Kochel *et al.*, 1991). The identities of p115 and p124 are unknown. One hypothesis to emerge from these analyses is that the transforming effect of v-*rel* may be due to its ability to bind to NF-κB or related DNA binding complexes and prevent their transcriptional function. This might explain why v-*rel* oncoproteins can apparently transform cells whether they are located in the nucleus or the cytoplasm (Gilmore and Temin, 1988; Hannink and Temin, 1989). For instance, it is striking that v-*rel* seems to preferentially transform the same cell lineage that exhibits constitutive NF-κB binding activity. This connection leads one to imagine that NF-κB could regulate something analogous to a tumor-suppressor gene that would inhibit the growth of mature B cells.

B. Cloning of the p50 and p49/p50B cDNAs

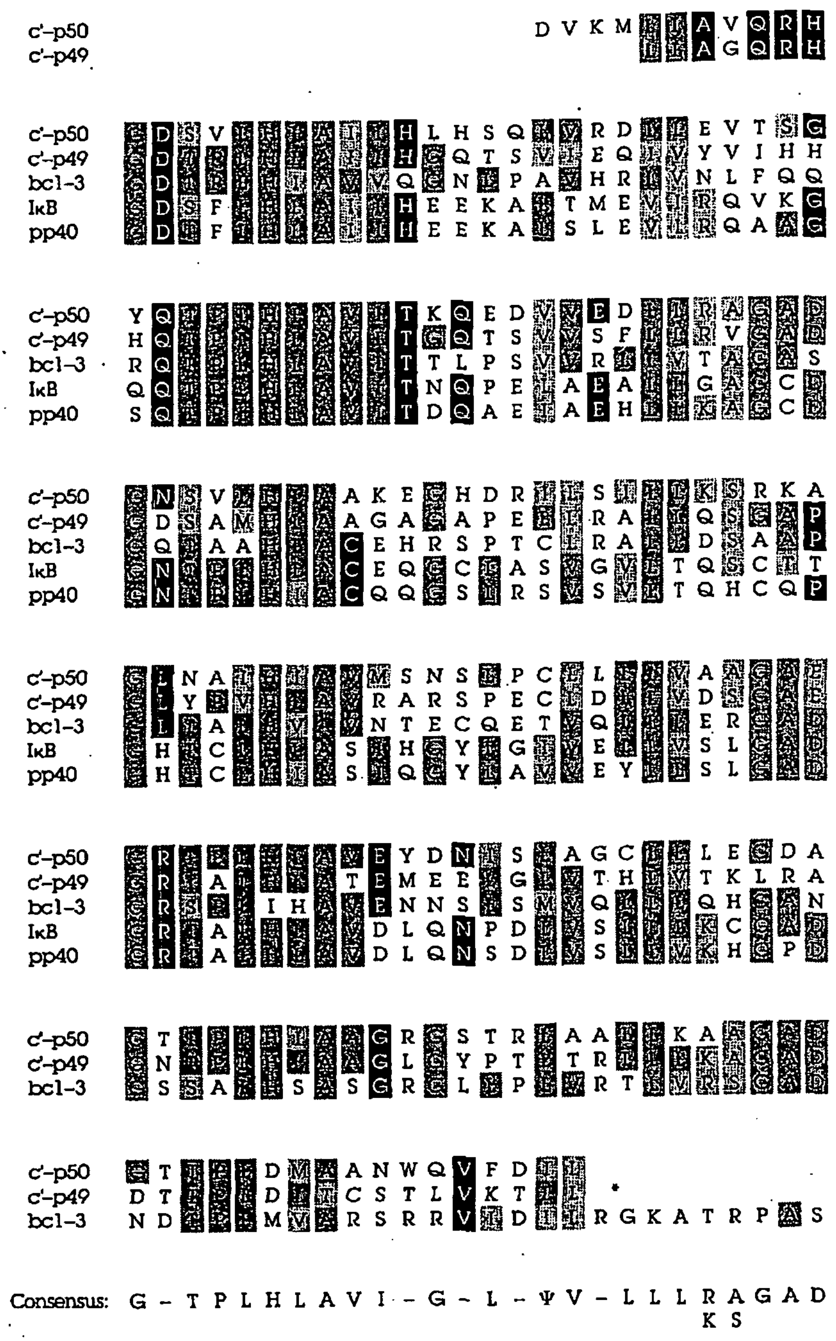
Though not initially recognized, the NF-κB p50 and p49/p50B subunits were first cloned in a collection of cDNAs isolated from a library made from mitogen-induced human peripheral blood mononuclear cells (Irving *et al.*, 1989; Zipfel *et al.*, 1989). The identity of these coding sequences was then conclusively established through independent cloning efforts based on protein microsequence from purified protein and binding analyses (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991; Schmid *et al.*, 1991). Evidence indicates that p50 could be the same as KBF-1, the constitutively active nuclear factor already known to bind NF-κB sites in the H2 gene (Kimura *et al.*, 1986; Israel *et al.*, 1987, 1989; Baldwin and Sharp, 1987; Kieran *et al.*, 1990). The p50 cDNA encodes for a 105-kDa protein, p105, that contains an N-terminal domain of 360 amino acids homologous to *rel*. The full-length protein product is unable to bind DNA. It needs to be proteolytically processed, near a stretch of glycine amino acids in the middle of the protein, to a mature form of approximately 50 kDa that includes the Rel domain. This has been found to occur by an ATP-dependent process that leads to the production of mature p50 and the rapid degradation of the C-terminal portion of the precursor protein (Fan and Maniatis, 1991). The C-terminal domain of 55 kDa that typically is cleaved off contains eight "ankyrin repeat" protein domains that can serve to keep the p50 moiety in a nonbinding form and prevent its nuclear localization (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990; Meyer *et al.*, 1991; Henkel *et al.*, 1992) (see Figs. 4 and 6). This may be a crucial regulatory interaction because in a B-cell lymphoma-associated translocation, a fusion gene has been created in which the Rel DNA binding domain of the p49/p50B gene, *lyt-10*, is preserved but the ankyrin repeat domain has been lost (Neri *et al.*, 1991). When independently expressed in bacteria, this portion of p105 is a potent inhibitor of DNA binding of p50 homodimers but not NF-κB or p65 homodimers (Liou *et al.*, 1992). In addition to specifying a previously undescribed DNA binding motif, the *rel* homology domain promotes dimerization. Thus, p50 forms homodimers or participates in heterodimer formation with p65 or other *rel* homology proteins (Kieran *et al.*, 1990).

The 4.0-kb mRNA for p105 is present in many tissues but can be upregulated by many of the same agents that promote the dissociation of NF-κB from I-κB (Bours *et al.*, 1990; Meyer *et al.*, 1991). An additional 1.9-kb mRNA encoding I-κBγ is also present in certain cells (Inoue *et al.*, 1992a). Phorbol ester stimulation of HL60 or HeLa cells or TNF-α stimulation of HL60 cells strongly induced the p105 mRNA. In human peripheral blood T cells, mitogenic stimuli such as PMA/PHA and calcium ionophore also elevate the p105 mRNA (Bours *et al.*, 1990). The mRNA is superinduced by inhibitors of protein synthesis. Interestingly, the immunosuppressive agent cyclosporin A strongly inhibited p105 mRNA induction by calcium ionophore but had only a modest effect on induction by PMA or PMA/PHA (Bours *et al.*, 1990). These findings give rise to the idea that sustained gene expression after various stimuli may be primarily due to increased synthesis of p105 mRNA and p50 protein (Hohmann *et al.*, 1991a). It has been recently shown that the p105 gene promoter is itself activated through an NF-κB binding site (Ten *et al.*, 1992).

Analysis of subcellular localization by both immunofluorescence and immunoprecipitation reveals that the p105 precursor is restricted to the cytoplasm, whereas processed p50 is both nuclear and cytoplasmic (Blank *et al.*, 1991). The sixth ankyrin repeat and adjacent acidic region of p105 were found to be important for cytoplasmic localization (see Fig. 6). Moreover, excision of the seventh ankyrin repeat or the acidic region between ankyrin repeats 6 and 7 allows DNA binding of p105. Thus, each ankyrin repeat in p105 may not function equivalently in causing subcellular localization or inhibition of DNA binding. Recent evidence indicates that p105, which is situated extranuclearly, can bind and thereby moor p65 in the cytoplasm (Rice *et al.*, 1992).

The function of the p50 subunit has been analyzed by cotransfection assays both on its own and as a partner to other subunits. It appears that p50 has little or no ability to cause transcriptional trans-activation through certain DNA sites that it binds (Kieran *et al.*, 1990), though when tested by *in vitro* transcription assays, p50 can function as an activator (T. Fujita and D. Baltimore, personal communication). In fact, p50 seems to suppress the activation of certain binding sites by p50/65 heterodimers (Schmitz and Baeuerle, 1991; Kang *et al.*, 1992a). KBF-1 has features of a transcriptional antagonist at κB sites (Israel *et al.*, 1989; Hemar *et al.*, 1991b). By contrast, p65 likely contains a trans-activating domain and may make p50/p65 heterodimers much stronger transcriptional activators (see below) (Nolan *et al.*, 1991; Ballard *et al.*, 1992). Interestingly, coexpression of the p65 and p50 subunits in Jurkat cells led to synergistic activation of κB-dependent transcription, but coexpression of p65 and the v-*rel* oncogene inhibited transcription (Ballard *et al.*, 1992).

Recently a cDNA has been cloned for an alternative DNA binding subunit of NF-κB that is very similar to p50 and which has been called variously p49 or p50B (Schmid *et al.*, 1991; Bours *et al.*, 1992). The full-length protein encoded is 97–100 kDa and, like p105, contains an N-terminal Rel domain and a C-terminal ankyrin repeat domain. The mRNA for p97/p100 is approximately 3.3 kb. The amino acid similarity between p97/p100 and p105 in the Rel region is ~60% identity but then drops off in the C-terminus to give an overall amino acid identity of ~40%. Schmid and co-workers have suggested that a cluster of histidine and cysteine residues in p49/p50B may chelate zinc in a functionally important way (1991). Just as for p105, the p97/p100 protein must be cleaved to an approximately 50-kDa form in order to specifically bind DNA. The mature form, p49/p50B, can heterodimerize with p65 and other Rel proteins and


c-p50
c-p49
bcl-3
IκB
pp40
Consensus: G - T P L H L A V I - G - L - Ψ V - L L L R A G A D
K S

binds κB sites with high affinity. When cotransfected with p65, p49/p50B can drive HIV-1 long terminal repeat (LTR) transcription but seems to have different functional effects depending on the exact sequence of the κB site with which it interacts (Schmid *et al.*, 1991; Perkins *et al.*, 1992).

C. The p65 Subunit

The cDNA isolated for the p65 NF-κB subunit reveals homology to the *rel* protein family (Nolan *et al.*, 1991; Ruben *et al.*, 1991) (Fig. 4). The cDNA clone corresponds to an mRNA of 2.6 kb that was detectable in all tissues tested and encoded a protein of 549 amino acids. The p65 mRNA does not seem to be increased very significantly by mitogenic agents that potently induce p50 (Ito *et al.*, 1992; U. Siebenlist, personal communication). As expected, the full-length protein from the cDNA clone could heterodimerize with p50 and together these subunits form a tight nucleoprotein complex with κB sequences. Thus, in contrast to p50, the p65 protein appears not to require cleavage from a larger precursor.

Studies aimed at the characterization of the different subunits have been focused mainly on the p50 subunit because of its strong DNA binding activity (Kawakami *et al.*, 1988; Baeuerle and Baltimore, 1989). However, p65, which originally appeared to be unable to bind DNA in mobility shift assays (Baeuerle and Baltimore, 1989) or by UV crosslinking (Kawakami *et al.*, 1988; Collart *et al.*, 1990), has now been shown to be a DNA binding subunit with important properties (Nolan *et al.*, 1991; Urban and Baeuerle, 1991). In addition to the ability of p65 to change the affinity of p50 for NF-κB binding sites, it may have potentially important transcriptional properties that differ from p50 (Baeuerle, 1991). A naturally occurring spliced variant of p65 has been shown to cause cellular transformation (Narayanan *et al.*, 1992).

Binding of the p65 homodimer can be completely inhibited by purified I-κBβ, indicating that p65 alone could directly interact with I-κBβ. These results were taken to suggest that a domain in the Rel homology region of p65 (which is missing in the corresponding part of p50) is responsible for interaction of I-κB with NF-κB complex. Interestingly, p65 is most highly related to the c-*rel* protein in sequence, especially the *rel*B variant (Ryseck *et al.*, 1992) (Fig. 5), and seems to contain a C-terminal region that is

FIG. 6 Comparison of protein sequences of the ankyrin repeats in proteins that have been demonstrated to inhibit the DNA binding of Rel/NF-κB family members. Dark grey shading indicates amino acids that are conserved in all repeats. Light grey shading indicates conservative substitutions in otherwise conserved positions. The black shading indicates amino acids that are the same within a given repeat as the protein sequences are aligned. A consensus for ankyrin repeats is shown at the bottom.

believed to be a transcriptional trans-activating domain of c-*rel* (Nolan *et al.*, 1991; Schmid *et al.*, 1991; Ballard *et al.*, 1992; Schmitz and Baeuerle, 1991; Ruben *et al.*, 1991, 1992). A region of the protein from amino acids 435 to 459 appears to be functionally important for activation. Notable features of this domain include negatively charged amino acids and a motif resembling a partial leucine zipper (Ruben *et al.*, 1992). p65 can form homodimers that can function as transcriptional trans-activators (Nolan *et al.*, 1991; Schmitz *et al.*, 1991). A spliced variant of p65 that has lost a protein region important for dimerization has been described in certain cell types (Ruben *et al.*, 1992).

D. Other Modes of Regulation

A *rel* homology protein that heterodimerizes but does not allow recognition of certain κB motifs would likely interfere with transcriptional activation of those sites. A precedent for this form of gene control comes from the protein named Id, which is a helix–loop–helix-type transcriptional regulator and inhibits gene activation by these proteins without binding to DNA itself (Benezra *et al.*, 1990; Sun *et al.*, 1991). This type of dominant negative interference has been demonstrated for a mutant version of the p50 protein that conserves dimerization ability but is unable to bind DNA (Logeat *et al.*, 1991). This mutant can block binding of other members of the Rel family *in vitro* and can act as a transcriptional dominant negative *in vivo*. Such a dominant negative inhibitor of p50, termed I-rel, has been reported recently (Ruben *et al.*, 1992).

In summary, patterns of protein–protein association have been clearly defined using polypeptides generated from cloned Rel/NF-κB subunit genes. These would seem to indicate multiple levels of interactive control to finely tune transcriptional responses (see Fig. 7). Regulation may result from the different combinatorial possibilities of p50 or p49/p50B when coupled to p65 or other *rel* homology proteins. All of the DNA binding subunits of NF-κB as well as Rel proteins may be controlled by release from cytoplasmic stores, though they seem to appear in the nucleus with different kinetics (Ballard *et al.*, 1990; Molitor *et al.*, 1990). Another level may emerge from the transcriptional effects that a given subunit combination generates at differing DNA sites in the repertoire of κB motifs (Sica *et al.*, 1992). A third level may result from interactions between Rel family members that do not involve DNA binding. There appears to be synergistic transcriptional effects of multiple binding sites for NF-κB that are not a function of cooperative binding (Pettersson and Schaffner, 1990).

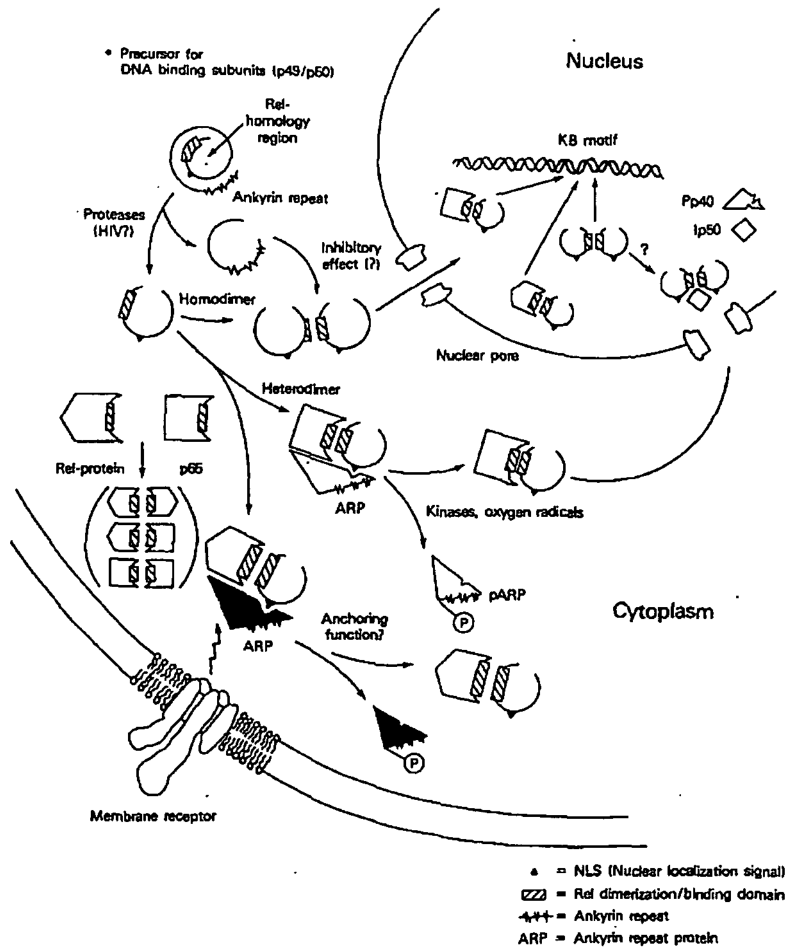


FIG. 7 A hypothetical scheme depicting several pathways of NF-κB and Rel regulation.

E. The Rel Homology Family Extends to *Drosophila*

v-*rel* and c-*rel* share a strong homology (47%) with the amino-terminal half of the *Drosophila dorsal* protein (Steward, 1987) (Fig. 5). The protein encoded by this maternal effect gene appears to govern the establishment of dorsal–ventral axis in the developing fly embryo (Ip *et al.*, 1991).

Importantly, the *dorsal* protein is regulated by partitioning between the cytoplasm and nucleus; this was an early clue that *dorsal* and NF-κB could be related (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Hunt, 1989). Relocalization of the dorsal protein from cytoplasm to the nucleus is crucial for its morphogenetic function (Steward, 1989). The protein region that is conserved between *dorsal* and the Rel family members is responsible for DNA binding and dimerization (Steward, 1987). Recently it has been shown that *dorsal* binds to κB motifs with high affinity (Ip *et al.*, 1991). The *dorsal* gene can exert a negative transcriptional effect on genes such as zerknüllt (*zen*) and decepentaplegic (*dpp*) but seems to activate other genes such as *twist* (Jiang *et al.*, 1991; Pan *et al.*, 1991). Also, the nuclear translocation and possibly DNA binding of the *dorsal* gene product may be inhibited by the protein product of the *cactus* gene much the same way that NF-κB and I-κB interact (see Hunt, 1989).

F. I-κB and Other Ankyrin Repeat Proteins

An important mechanism for controlling NF-κB binding activity is provided by protein–protein interactions that involve I-κB-like molecules. A cDNA clone has been recently isolated that encodes a 36-kDa protein (318 amino acids), called MAD3, that appears by several criteria to be identical to I-κBβ (Haskill *et al.*, 1991; I. Verma, personal communication). The MAD3 protein is the appropriate molecular weight and is a highly specific inhibitor of NF-κB DNA binding but not KBF-1. In addition, the sequences of two peptides derived from purified I-κBα are found in the coding sequence of the MAD3 clone (Davis *et al.*, 1991). Also, the MAD3 protein and NF-κB could be coimmunoprecipitated with antibody against NF-κB, indicating a direct interaction between the two proteins (Haskill *et al.*, 1991). Interestingly, the mRNA for MAD3 is rapidly induced during the process of adherence of monocytes to plastic. A homologous rat protein is rapidly induced during liver regulation following partial hepatectomy (Tewari *et al.*, 1992a,b). Together these results suggest that I-κBβ might be part of a negative feedback mechanism to limit NF-κB activation. As might be expected for I-κB, the MAD3 protein also exhibits a putative PKC phosphorylation site near its C-terminus (Haskill *et al.*, 1991).

One of the most significant features of the MAD3 protein is that it contains five tandem repeats of the ankyrin motif (see Fig. 6). As mentioned, this repeat had also been observed in multiple copies in the C-terminal region of the p105 protein precursor to p50 and the p97 precursor to p50B (Kieran *et al.*, 1990; Ghosh *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991). The ankyrin repeat had been identified as a motif that was found in human erythrocyte protein ankyrin and then also observed

in proteins required in cell cycle control and tissue differentiation (for a review see Lux *et al.*, 1990). Because both MAD3 and the C-terminal portions of p105 and p97 had the ability to inhibit the binding of p50 to DNA, these ankyrin repeats were thus implicated in the interaction between NF-κB and I-κB. A comparison of several ankyrin repeat proteins (ARPs) revealed that the ankyrin repeat seems to be the sole domain in common (G. Nolan, personal communication) (see Fig. 6).

The realization that NF-κB is a member of the *rel* homology family prompted an investigation of the functional properties of the pp40 *rel*-associated protein (Kerr *et al.*, 1991). pp40 is a phosphoprotein that is complexed with Rel proteins (Tung *et al.*, 1988; Simek and Rice, 1988). It can be detected in both the cytoplasm and the nucleus of v-*rel*-transformed cells (Simek and Rice, 1989; Davis *et al.*, 1990, 1991) and the B-lymphoma lines WEHI 231 and 70Z/3 (Kerr *et al.*, 1991). pp40 associates with the amino terminus of v- and c-*rel* (Simek and Rice 1988; Davis *et al.*, 1990, 1991); it is reminiscent of I-κB because of both its molecular size and the ability of phosphorylation or detergent to diminish its binding to *rel* proteins. Biochemical studies determined that purified pp40 would inhibit DNA binding of *rel* homology proteins. Intriguingly, pp40 and I-κBβ inhibit DNA binding of p50/p65, p50/c-*rel* complexes but not p50 homodimers. (Kerr *et al.*, 1991; Davis *et al.*, 1991). Tryptic peptide mapping indicates that pp40 may, in fact, be identical to I-κBβ. The distribution of pp40 would suggest that the activation loops for complexes involving *rel* proteins and ARPs have the potential to be taking place in both the nucleus and the cytoplasm.

The sequence of a cDNA for pp40 has been recently reported by Davis and colleagues (1991). The pp40 coding sequence contains five ankyrin repeats just as for other proteins that inhibit the binding of Rel family members (see Fig. 6). This strengthened the idea of a gene family of ARPs that have properties akin to those of I-κB. pp40 also contains potential casein kinase II and tyrosine kinase sites (Davis *et al.*, 1991).

Recent work has led to the molecular characterization of a third form of I-κB, termed I-κBγ (Inoue *et al.*, 1992a). This form is derived from a 2.5-kb mRNA that is an apparent splice product of the p50 gene that appears in lymphoid cells. The encoded 607-amino acid protein (70 kDa) corresponds to the C-terminus of the p105 precursor protein that contains ankyrin repeats. I-κBγ prevents NF-κB, p50 homodimer, and c-*rel* from binding to DNA. Furthermore, it has been shown to prevent transit to the nucleus and gene activation by c-*rel*. The protein sequence also contains a consensus protein kinase A phosphorylation site (RKLS), indicating the possibility that it too is regulated by phosphorylation.

A putative leukemia oncogene, Bcl-3, also appears to have I-κB-like properties (Hatada *et al.*, 1992; Wulczyn *et al.*, 1992). Bcl-3 is an ARP encoded at the locus of the chromosomal translocation t(14:19)(q32;q13.1) that occurs in cases of chronic lymphocytic leukemia and has seven copies of the ankyrin repeat (Ohno *et al.*, 1990). In contrast to I-κBα or β, Bcl-3 may preferentially inhibit p50 homodimers and NF-κB, but not p65 homodimers. In an important link to previous studies that have implicated various *rel* proteins in cell proliferation, the expression of Bcl-3 increases *in peripheral blood mononuclear cells following mitogenic stimulation* (Ohno *et al.*, 1990; Franzoso *et al.*, 1992). The ankyrin repeats in this protein bear a striking resemblance to those found in the C-terminal region of the p105 protein. Ankyrin repeats are likely to differ in their function depending on their precise amino acid sequence, thus affording another venue for specifity in the interactions between the *rel* and the ARP families.

The function of the ankyrin repeat is not completely understood. Usually *proteins, unless very small, cannot pass freely in the nucleus and need to* carry specific basic sequences called nuclear localization signals (NLS) exposed on the protein surface (for review see Dingwall and Laskey, 1986; Nigg *et al.*, 1991). Such a sequence has been shown to be important for the nuclear accumulation of processed p50 (Blank *et al.*, 1991). One hypothesis was that the ankyrin repeat could either mask the NLS or anchor in some way the *rel* protein to cytoskeletal or cellular membrane components such that the NLS would not function (Ghosh *et al.*, 1990). I-κB not only prevents DNA binding activity when associated to NF-κB in the cytosol but also apparently blocks nuclear translocation (Baeuerle and Baltimore, 1988a,b; Henkel *et al.*, 1992; Beg *et al.*, 1992). This has also been seen clearly in the cytological localization of the *dorsal* protein (Hunt, 1989). An alternative view is that the ankyrin repeat is really a protein–protein interaction domain that yokes the *rel* homology proteins to ARPs (Thompson *et al.*, 1991) and allows cytoplasmic localization secondarily, possibly through other domains. The latter conjecture is given some credence by the fact that the pp40 : *rel* complexes are found in the nucleus as well as the cytoplasm (Davis *et al.*, 1990, 1991).

The model of cytoplasmic localization by the union of NF-κB and I-κB is very similar to the regulation of the glucocorticoid receptor (Picard and Yamamoto, 1987). The glucocorticoid receptor is cytoplasmic in the absence of glucocorticoids, and its NLS is presumably covered by the binding of heat shock protein 90 (hsp90; Picard and Yamamoto, 1987). Steroid binding to the receptor could overcome inhibition by dissociating the receptor and hsp90. Recent evidence suggests that the regulation is *more complicated since the receptor bound to hsp90 is not necessarily* excluded from the nucleus—a feature also observed in complexes between *rel* and pp40 (Sanchez *et al.*, 1990). The major difference between the glucocorticoid system and NF-κB is that in the case of the glucocorticoids the allosteric modification that results in nuclear translocation affects the transcription factor itself and not the "anchoring" protein.

Finally, it is intriguing that the precursors for p50 and p49/p50B couple both a *rel* homology domain and an ARP in a single bifunctional polypeptide (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Bours *et al.*, 1990, 1992; Meyer *et al.*, 1991; Schmid *et al.*, 1991). p50 and p49/p50B are, so far, the only members of the Rel homology family that exist in the form of precursors. Many roles can be envisioned for the ankyrin repeats that are *located in the C-terminal portion of these proteins. Most intriguing is the* possibility that cleavage of the mature form of the binding protein from the precursor is a critical step in the regulation of NF-κB binding. The process requires ATP, and the ankyrin repeats are not required for processing (Fan and Maniatis, 1991). Because of the strong DNA binding ability of p50, this proteolytic event may represent a mechanism to govern the preponderance of p50 in homodimeric versus heterodimeric form. This *could be crucial since p50 homodimers appear to be unaffected by* I-κBβ that would result in cytoplasmic localization of NF-κB through its interaction with p65.

VIII. NF-κB and Viruses

Aside from the obvious role of the Rel protein as the transforming gene product of the Rev-T virus, NF-κB and potentially other Rel proteins have been found to play a role in transcriptional regulation of many other viruses. Most work to date has been done on retroviruses that infect human T lymphocytes—the human immunodeficiency viruses and the human T lymphotropic virus, type I. Several other viruses have also been studied and will be discussed: adenovirus; SV-40; hepatitis B virus; herpesvirus types I and VI and their relative, cytomegalovirus. The characteristic rapidity of NF-κB induction can be utilized by viruses as a strategic tool to initiate its reproduction or to place its life cycle under the control of the host cell.

A. Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a retrovirus that infects $CD4^+$ T lymphocytes and some monocytes causing acquired immunodeficiency syndrome (AIDS) (Cullen and Greene, 1989). *The role of NF-κB in controlling* the HIV long terminal repeat (LTR) was first established by Nabel and Baltimore, who demonstrated a direct correlation between increases in NF-κB binding and the transcriptional activity of the LTR following activation of T cells (1987). Deletion analysis of the HIV LTR had established that a strong enhancer element within the LTR was centered over a nucleotide sequence that contained two homologies to the Ig-κ gene NF-κB site (Muesing *et al.*, 1987). Most isolates of HIV-1 and simian immunodeficiency virus (SIV) have two functional NF-κB sites in the LTR, but in HIV-2 there is conservation of only one of the NF-κB binding sites (Dewhurst *et al.*, 1990; Anderson and Clements, 1991; Novembre *et al.*, 1991).

The possibility of a strong association between T-cell activation and expression of the HIV LTR has come to dominate thinking about the regulation of the life cycle of HIV. A characteristic feature of HIV infection is that fulminant disease often occurs many months or even years after the initial infection (Haseltine, 1988; Cullen and Greene, 1989). This delay in viral pathogenesis, called the latent period, could be due to the residence of a provirus in a small population of T lymphocytes in a quiescent state (reviewed in Garcia-Blanco and Cullen, 1991). Activation of those T lymphocytes by antigen stimulation could increase NF-κB and lead to transcriptional expression of the provirus and active production of the virus (Rosen *et al.*, 1985; Zagury *et al.*, 1986; Nabel and Baltimore, 1987; Harada *et al.*, 1988; Tong-Starksen *et al.*, 1989). Other inducers of NF-κB, such as TNF-α and IL-1 also could increase virus production (Folks *et al.*, 1989; Osborn *et al.*, 1989). This reawakening of the virus would presumably cause decreased numbers of the CD4$^+$ cells leading to the immunodeficiency syndrome.

A correlation between the induction of NF-κB and LTR transcription has received considerable experimental support. A variety of studies show that induction of NF-κB in either T lymphocytes or monocytes causes increased transcriptional activity of the HIV LTR (Nabel and Baltimore, 1987; Garcia *et al.*, 1987; Franza *et al.*, 1987; Kaufman *et al.*, 1987; Bielinska *et al.*, 1989; Griffin *et al.*, 1989, 1991; Zeichner *et al.*, 1991; Berkhout and Jeang, 1992; Hillman *et al.*, 1992). Moreover, LTR activity does not seem to be restricted to cells such as CD4$^+$ T cells and monocytes that normally manifest productive infection but may depend more on the availability of NF-κB. If the LTR is tested in B cells, NF-κB stimulates transcription strongly and B cells that artificially express the CD4 surface molecule permit HIV replication quite well (Calman *et al.*, 1988). T cells that do not express the p50/p65 heterodimer have restricted HIV expression (Raziuddin *et al.*, 1991). Methylation of cytosine nucleotides can inhibit NF-κB binding, suggesting that a methylated HIV provirus may be resistant to activation (Bednarik *et al.*, 1991). Other viruses that can activate NF-κB such as herpesvirus and cytomegalovirus and the c-Ha-*ras* oncogene can cause transactivation of the HIV LTR through the NF-κB binding site and lead to viral activation (Ensoli *et al.*, 1989; Horvat *et al.*, 1991; Arenzana-Seisdedos *et al.*, 1989; Geng *et al.*, 1992). Curiously, one study found that antisense oligonucleotides complementary to the NF-κB binding motif interfere with trans-activation of the LTR (Laurence *et al.*, 1991).

Most studies on the transcriptional control of HIV have been carried out by transiently transfecting cells with plasmids containing the HIV LTR linked to various reporter genes (usually bacterial chloramphenicol acetyltransferase). These artificial gene constructs may only partly reflect the normal transcriptional control of HIV replication. Thus it became important to also test the infectivity of HIV when various *cis*-acting elements in the LTR have been mutated to gain an independent assessment of the role of these sequences in the virus life cycle. Studies of HIV replication in response to cytokines found an association between induction of NF-κB binding and transcriptional activation of the virus (Hemar *et al.*, 1991a; Israel *et al.*, 1989; Rosenberg and Fauci, 1990). One study found that viruses containing deletions of both NF-κB sites were still infective in MT-4 cells (Leonard *et al.*, 1989). Further analysis revealed that the two NF-κB sites and three Sp1 sites were functionally redundant and not all required for infectivity (Ross *et al.*, 1991). Various alterations of these sites seemed to change the cell type that permitted virus production as well as the replicative and cytopathic properties of HIV-1 (Ross *et al.*, 1991; Englund *et al.*, 1991; Parrott *et al.*, 1991). This contrasts with the complete loss of viral activity seen when the TAR region was altered (Leonard *et al.*, 1989). Moreover, specific NF-κB inducers can stimulate virus replication but the effect varies with cell type—TNF-α can induce virion production in certain T-cell lines but not others and LPS will apparently stimulate HIV production in monocytic/macrophage lines but not T cells (Matsuyama *et al.*, 1989; Pomerantz *et al.*, 1990). Thus viral replication seems to involve multiple levels of control of which NF-κB is only a part.

Recent work has more fully assessed the transcriptional control of the HIV LTR and has found transcriptional induction mechanisms that are independent of NF-κB. One early report identified an 86-kDa protein (HIVEN 86A) that interacted strongly with the HIV enhancer, which is now believed to be the c-Rel protein (Franza *et al.*, 1987; Ballard *et al.*, 1990,1992). Other biochemical studies have described interactions of proteins with the LTR enhancer elements that were the same size as NF-κB but had some distinctive features (Wu *et al.*, 1988a,b). Moreover, LTR transcription seems to also depend on factors that bind sites outside of the enhancer comprised of NF-κB sites, such as factor Sp1, which has three binding sites, potentially the factor NF-AT as well as other factors (Jones *et al.*, 1986; Lu *et al.*, 1991). Finally, interesting evidence has emerged that enhancer-independent inducibility of the HIV LTR may involve other proteins binding in close proximity to the TATA motif (Jones, 1989; Sakaguchi *et al.*, 1991; D. Margolis and S. Strauss, personal communication). The participation of factors other than NF-κB has been recently demonstrated in nontransformed T lymphocytes—phorbol ester or antigen were able to induce both NF-κB and LTR transcription but TNF-α and antibodies against CD3 chains induced NF-κB and not LTR transcription (Hazan *et al.*, 1990). Also, c-*rel* may be an activator of the LTR (Muchardt *et al.*, 1992).

Other new findings introduce further considerations into the model that assumes NF-κB primarily controls whether HIV becomes transcriptionally "latent" or productively infects a target cell (Nabel, 1991; Doppler *et al.*, 1992). Recent work has shown that HIV infection causes induction of NF-κB in monocytic cell lines that typically only have very low levels of this factor (Bachelerie *et al.*, 1991; Neuvent *et al.*, 1991; Suzan *et al.*, 1991; Roulston *et al.*, 1992). This suggests that NF-κB is involved in an amplification loop that contributes to mantaining productive infection. Other work suggests that a protease encoded by the virus can cleave the p105 precursor to activate binding of the p50 subunit of NF-κB (Riviere *et al.*, 1991). Therefore, infection by the virus alters the host cell by inducing NF-κB, thereby making the environment more hospitable for its own replication. To adhere to the original model of transcriptional latency, a means of silencing NF-κB driven LTR transcription following infection must also be contemplated.

B. HTLV-1 and NF-κB

HTLV-1 is the etiological agent of the adult T-cell leukemia (ATL) (Yoshida and Seiki, 1987; Wong-Staal *et al.*, 1983, reviewed in Broder and Gallo, 1985). The cellular and molecular basis of HTLV-1 transformation is not completely understood but one protein, Tax, of the three proteins encoded by the pX region of the HTLV-1 genome has been strongly implicated in oncogenesis (Nerenberg *et al.*, 1987; Pozzati *et al.*, 1990). Tax is a transcriptional trans-activator but does not bind to DNA or stimulate the activity of *cis*-acting control elements directly. Its effects are independent of new protein synthesis (Jeang *et al.*, 1988). It appears to transmit activation signals through other transcription factors including NF-κB (Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988; Cross *et al.*, 1989; Ruben and Rosen, 1990; Mauxion *et al.*, 1991). The Tax protein appears to be the only component of HTLV-1 that is necessary and sufficient to induce NF-κB, but this is not completely clear (Arima *et al.*, 1991; LaCoste *et al.*, 1991). By using NF-κB and other transcription factors to trans-activate a set of genes that could foster T-cell proliferation, infected cells could be predisposed to additional mutations that would cause outright transformation (Yoshida and Seiki, 1987).

Consistent with this hypothesis, the Tax protein of HTLV-1 and the HTLV-2 trans-activator protein activate genes involved in T-cell growth (Greene *et al.*, 1986; Wano *et al.*, 1988). The genes for both IL-2 and the IL-2 receptor α chain are stimulated by Tax-induced NF-κB (Inoue *et al.*, 1986; Cross *et al.*, 1987; Maruyama *et al.*, 1987; Siekevitz *et al.*, 1987a; Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988; Bohnlein *et al.*, 1989b; Hoyos *et al.*, 1989). Other genes characteristically expressed following antigen stimulation of T cells are also up-regulated by Tax-induced NF-κB, including c-*fos* (Fujii *et al.*, 1988), GM-CSF (Green *et al.*, 1989; Schreck and Baeuerle, 1990), IL3 (Miyatake *et al.*, 1988), the β-interferon gene (LeBlanc *et al.*, 1990), and lymphotoxin (Paul *et al.*, 1990). In addition to contributing to the process of neoplasia, these NF-κB-stimulated genes may cause other sequellae in HTLV-1-infected individuals. One life-threatening complication of ATL is hypercalcemia, which may be the result of bone resorption due to the osteoclast-activating properties of lymphotoxin (Paul *et al.*, 1990). Tax also activates other NF-κB-responsive enhancer elements such as the PRD II motif in the β-interferon gene (LeBlanc *et al.*, 1990). Tax deregulation of NF-κB therefore sets in motion a genetic program that causes malignancy and other pathological consequences.

Tax is also a potent trans-activator of the HTLV-1 LTR (Sodroski *et al.*, 1984; Felber *et al.*, 1985; Seiki *et al.*, 1986; Fujisawa *et al.*, 1985) as well as the viral enhancers from HIV-1 and SV-40 (Siekevitz *et al.*, 1987b; Bohnlein *et al.*, 1988, 1989a; Marriott *et al.*, 1990; Miyatake *et al.*, 1988; Nakamura *et al.*, 1989; Zimmerman *et al.*, 1991). Interestingly, Tax transactivation of its own LTR depends on a promoter region that binds constitutively expressed proteins like CREB and Jun as well as NF-κB (Yoshimura *et al.*, 1990; Xu *et al.*, 1990; Numata *et al.*, 1991). The ability of Tax to transactivate the HIV retrovirus most likely occurs through NF-κB. HTLV-1 could potentially exacerbate the progression of AIDS.

The mechanism by which Tax regulates NF-κB is unclear. Tax expression in the cell leads to greater NF-κB binding activity and may require other cellular proteins (Ballard *et al.*, 1988; Leung and Nabel, 1988; Ruben *et al.*, 1988, 1989; Cross *et al.*, 1987). Tax has been found to increase the level of mRNA for both c-*rel* and the p50 subunit of NF-κB (Arima *et al.*, 1991). It is also likely that Tax releases NF-κB from the cytoplasmic complex with IκB, but this is difficult to reconcile with the fact that Tax is typically a nuclear protein (Goh *et al.*, 1985; Kiyokawa *et al.*, 1985). Strikingly, soluble Tax protein can be taken up by tissue culture cells and activate NF-κB (Lindholm *et al.*, 1990, 1992). In this situation, Tax is found in both the nucleus and the cytoplasm. Because NF-κB is not the only transcription factor induced by Tax, it is interesting to know whether different parts of the protein stimulate distinct transcription factors. In fact, by placing missense mutations in the Tax coding regions, Smith and Greene have shown that different domains are utilized to activate genes through the NF-κB or CREB/ATF pathways (1990).

C. Other viruses using NF-κB

Several studies have shown that cytomegalovirus uses NF-κB to stimulate its own transcription. Five potential NF-κB binding sites can be found in the defined enhancer region of the virus (Henninghausen and Fleckenstein, 1986). The NF-κB sites in the cytomegalovirus enhancer were found to be important for the transcription of the viral immediate early 1 gene (ie1) (Nelson and Groudine, 1986; Ghazal *et al.*, 1988; Cherrington and Mocarski, 1989). Moreover, the protein product of the ie1 gene was found to activate the binding of NF-κB and thereby autoregulate the expression of its own gene (Sambucetti *et al.*, 1989). Similar to the paradox of Tax activation of NF-κB, ie1 is a nuclear phosphoprotein and therefore it is unclear whether it could directly release NF-κB from its cytoplasmic complex with IκB.

Other DNA viruses such as herpes simplex virus and human herpes virus 6 appear capable of inducing NF-κB through virally encoded trans-activating proteins and may themselves be regulated by NF-κB (Gimble *et al.*, 1988; Ensoli *et al.*, 1989; Martin *et al.*, 1991). These viruses along with cytomegalovirus are frequently carried by HIV-infected individuals and could provoke the progression of AIDS by their ability to stimulate HIV expression through NF-κB (Ensoli *et al.*, 1989; Lusso *et al.*, 1989). Also potentially important for the pathogenesis of AIDS is the fact that a trans-activator from the hepatitis B virus, the X gene, uses NF-κB to stimulate transcription of the HIV LTR, cellular genes such as MHC class I, and other viral control regions (such as the SV-40 enhancer) (Twu *et al.*, 1989a,b; Twu and Robinson, 1989; Siddiqui *et al.*, 1989; Faktor and Shaul, 1990; Zhou *et al.*, 1990; Luber *et al.*, 1991; Lucito and Schneider, 1992; Meyer *et al.*, 1992). Adenovirus-5-transformed cells have high levels of nuclear NF-κB and strongly express the β-IFN and MHC class I genes (Nielsch *et al.*, 1991). NF-κB has also been implicated in the lymphoid-specific transcription of the E3 promoter of adenovirus (Williams *et al.*, 1990) and the core enhancer of SV-40 (Kanno *et al.*, 1989; Macchi *et al.*, 1989; Phares and Herr, 1991). The organization of viral control elements, such as for SV-40, is extremely compact and appears to contain binding sites for a great variety of cellular transcription factors, perhaps to allow expression in many cell types (Fromental *et al.*, 1988).

IX. Other Proteins That Bind κB Motifs

The widespread use of NF-κB in gene regulation has engendered a plethora of studies that describe a large number of κB site–protein complexes. One unexpected turn in our emerging understanding of NF-κB was the finding that two different protein structures are capable of high affinity recognition of κB motifs—the Rel domain and the zinc-finger domain. The Rel domain appears to contain distinct subdomains that are involved in DNA binding and dimerization.

Molecular cloning studies have identified multiple genes that contain each domain. Other studies have been completely descriptive and essentially survey the size of proteins that form high affinity interactions with a particular κB DNA sequence using either electrophoretic mobility shift or UV crosslinking assays. For example, Shakov *et al.* (1990) and Xanthoudakis and Hiscott (1990) describe sets of proteins that bind to the κB binding sequences in the TNF-α and the β-interferon genes, respectively. After detailed characterization by molecular cloning or biochemical analysis, some proteins have been found to have properties of special interest. These include MBP-1/PRDII BFI/HIV-EBP-1; beta; and H2-TF1.

A. MBP-1/PRDII/HIV-EBP-1: A Large Zinc-Finger Protein

MBP-1/PRDII BF1/HIV-EBP-1 is a protein that bears a zinc-finger DNA binding motif that has been cloned and analyzed by several different groups. It was originally identified by screening a bacteriophage expression library using the mouse MHC class I (H-2K^b) enhancer site as a probe (Singh *et al.*, 1988). Because of some combination of its affinity, molecular structure, and abundance, it has been cloned from bacteriophage expression libraries also with the PRDII motif from the β-interferon gene and a κB motif from the HIV enhancer (Fan and Maniatis, 1990; Nomura *et al.*, 1991). Since it displayed high affinity for κB sites, it was initially believed to be a clone for NF-κB itself. The gene for MBP-1/PRDII-BFI/HIV-EBP-1 has been mapped to human chromosome 6p22.3-p24 (Gaynor *et al.*, 1991). Several other κB binding proteins bearing a zinc-finger DNA binding domain that are homologues to MBP-1/PRDII-BFI/HIV-EBP-1 have also been cloned. These are HIV-EBP-2 (Nomura *et al.*, 1991), AT-BP1 and AT-BP2 (Mitchelmore *et al.*, 1990), MBP-2 and KBP-1 (Rustgi *et al.*, 1990), AGIE-BP-1 (Ron *et al.*, 1991), and αA-CRYBP1 (Nakamura *et al.*, 1990). Recent evidence suggests this is a large gene family (Ron *et al.*, 1991). Some of these have been postulated to play roles in specific genes such as αA-CRYBP1 in regulating expression of the α1-crystallin gene (Nakamura *et al.*, 1990).

A key feature of certain members of this family of zinc-finger domain genes is that they specify very long mRNAs (9–10 kb) and encode proteins of immense size (200–300 kDa) (Maekawa *et al.*, 1989; Fan and Maniatis, 1990; Nomura *et al.*, 1991). These are probably the largest DNA binding transcription regulatory proteins known. One of the remarkable features of these proteins is that they contain zinc-finger domains that are separated by a stretch of 50 kDa of intervening protein sequence (Fan and Maniatis, 1990; Baldwin *et al.*, 1990). The zinc-finger domains, though differing in primary sequence, display very similar, if not identical, binding specificities (Baldwin *et al.*, 1990). Also interesting is that the mRNA for these genes is induced by mitogens in T-lymphoma cells or virus and serum in fibroblasts (Baldwin *et al.*, 1990; Fan and Maniatis, 1990). Some members of this gene family appear to be highly expressed in brain (Nomura *et al.*, 1991).

B. Beta: A Brain-Specific Regulator

Analysis of nuclear protein extracts from various regions of the rat brain for binding to the Ig κB binding site revealed a novel mobility shift complex that migrates more slowly than NF-κB (Korner *et al.*, 1989). The complex was not found in extracts from a variety of visceral organs and was detectable in the gray but not the white matter of the brain. It is notable that the proenkephalin gene is highly expressed in these tissues and a binding site for the beta protein is found in the promoter of this gene. Moreover, it was shown that κB site-dependent reporter constructs were active when transfected into primary cultures of cerebeller neurons or astrocytes. Because of its restriction to certain areas of the brain and large apparent size on mobility shift, it may be derived from one of the zinc-finger protein genes whose mRNA is highly expressed in the brain (Nakamura *et al.*, 1990).

C. H2-TF1: A Putative MHC Class I Regulator

Another factor that has been characterized biochemically but has remained elusive in its molecular identity is H2-TF1. Originally identified by Baldwin and Sharp (1987), this factor bound to the palindromic κB motif (5′-TGGGGATTCCCCA-3′) located in the H-2K^b gene and made base contacts that were distinct from those made by NF-κB. Though it has been suggested that H2-TF1 and p50 are identical (Baeuerle, 1991), UV cross-linking experiments indicate that the H2-TF1 complex is due to a 125-kDa protein (R. Scheinman and A. Baldwin, unpublished results). The DNA site bound by H2-TF1 contributed to the basal activity and α-interferon inducibility of this class I promoter and was found to be conserved in a number of other class I genes (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Hakem *et al.*, 1989; Ackrill and Blair, 1989). Interestingly, the level of an H2-TF1-like binding protein and the activity of the MHC class I gene enhancer are suppressed by N-*myc* in rat neuroblastoma tumor cells. This seems to be due to suppression of a particular isoform of protein kinase C by N-*myc* (Lenardo *et al.*, 1990; Henseling *et al.*, 1990; Bernards, 1991).

Other proteins that have some binding specificity related to NF-κB include a binding protein to the MHC class II promoter (Gravallese *et al.*, 1989), a protein called KBF-2 that is detected in undifferentiated teratocarcinoma cells (Israel *et al.*, 1989); a protein binding to the HIV enhancer (named EBP-1, but of unknown relationship to the cloned zinc-finger protein named HIV-EBP1) (Clark *et al.*, 1988, 1990; Clark and Hay, 1989); and NF-GMa, a regulator of the expression of the *GM-CSF* gene (Shannon *et al.*, 1988). One recent report suggests the NF-AT can bind to certain NF-κB sites (McCaffrey *et al.*, 1992).

X. **Conclusions**

In addition to giving the overall picture of NF-κB in gene regulation, we hope we have also illuminated the weave of this rich tapestry of discovery by a large number of investigators. Knowledge of the various DNA binding proteins in this system has emerged from two long-standing parallel lines of investigation on NF-κB and the *rel* oncogene. Gene cloning has recently brought these fields together to the enrichment of both. Amino acid sequence similarities suggest that the NF-κB/Rel/*dorsal* system arose early in evolution to assume a variety of roles in gene regulation among both vertebrates, invertebrates, and possibly in yeast (Toyama *et al.*, 1992). Importantly, the wide utilization of NF-κB and its congeners in gene control indicates that work in this area will significantly influence many areas of molecular biology in the future.

In closing, it is important to note that studies on NF-κB have the ability to provide new and intriguing answers to old questions in gene regulation. An important paradox in the notion that proteins regulate genes is that the expression of the gene-regulatory proteins would seem to require an infinite regression of gene control proteins. NF-κB illustrates a transactivation mechanism that does not require new protein synthesis and thus can be directly induced independently of the transcription of its own gene. Another question has been how signal pathways connect the cell membrane to the nucleus. NF-κB itself can convey a signal from the cytoplasm directly to gene control elements in the nuclear DNA. By transmitting signals across the cell, NF-κB acts as a unique type of second messenger specifically designed for gene regulation. Certain other regulatory questions remain unexplained. We do not understand how NF-κB binding sites and transcriptional initiation sites that are separated by widely varying lengths of DNA communicate with each other within chromatin. Another mystery yet unsolved is how the same regulatory factors can uniquely activate various sets of genes in response to selected stimuli. This phenomenon hints at an information system for controlling RNA synthesis that will be far more complex and flexible than the genetic code that governs protein synthesis.

Acknowledgments

The authors thank Warren Leonard, Sergei Nedospasov, Marc Reitman, Nancy Rice, and Uli Siebenlist for helpful comments on the manuscript; Dr. David Baltimore, in whose laboratory some of the experiments depicted were carried out; and many investigators in the field who generously shared their ideas and unpublished findings. Jason J.-S. Chiu was supported by the Howard Hughes Medical Institutes–NIH Research Scholarship program. M.J.L. was supported in part by a Cancer Research Institute Investigator Award.

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Annu. Rev. Cell Biol. 1994. 10:405–55
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STRUCTURE, REGULATION AND FUNCTION OF NF-κB

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KEY WORDS: IκB, Rel, dorsal, ankyrin motif, gene regulation, immune activation, HIV cytokines

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INTRODUCTION

Survival of all life forms requires dynamic response to environmental change and challenge. In higher organisms such phenomena include defensive response to stress, injury, viruses, and pathogens. To mount effective responses, an organism requires a sensitive and rapidly acting system to detect and fight such potentially life-threatening circumstances. In the case of viruses and other microbiological pathogens, for example, the immune system is capable of recognizing these agents as foreign by their antigenic properties. To fight them, various immune cells are activated, differentiated, expanded, and summoned to relevant sites. Antibodies are synthesized and cell-mediated defenses activated. To achieve this, recognition of an invading agent by T cells, B cells, macrophages, and endothelial cells must elaborate defined genetic programs, such as the induced synthesis of soluble mediators (cytokines, growth factors, chemokines) and their receptors. The genetic response, i.e. the signal-responsive induction of gene expression, is mediated ultimately by factors that control the transcription of these genes. Among the various factors that contribute to the induction of these rapid-response genes, the transcription factor NF-κB stands out as a central, coordinating regulator. A vast and interrelated array of defense genes is regulated, at least in part, by a family of closely related dimeric complexes collectively referred to as NF-κB. Indeed, the association between induced cellular defense genes and NF-κB transcription factors dates at least as far back as insects in evolutionary terms.

Beyond playing a central role in defensive responses, NF-κB may modulate gene expression in various other situations that signal rapid gene expression. NF-κB and its regulators appear to function in growth control, as evidenced by mis-regulation of NF-κB genes encoding genes in certain tumors. A developmental role is indicated by the dorsal protein, a *Drosophila* homologue of vertebrate NF-κB factors: dorsal controls axial polarity in developing embryos in response to a positional cue.

The diversification of NF-κB during evolution into multiple genetically encoded polypeptides that assemble as homo- and heterodimers, and that bear unique and overlapping functional activities, as well as unique and overlapping expression patterns, portends wide and varied functional roles for this family of transcription factors. In addition to its role in expression of host cell genes, NF-κB has been usurped by various viruses, including the human immunodeficiency virus, to mediate viral gene expression.

NF-κB's most obvious characteristic is its rapid translocation from cytoplasm to nucleus in response to extracellular signals. Under most circumstances, these factors lie dormant in the cytoplasm of unstimulated cells, kept there by an inhibitory protein termed IκB. Many signals inactivate the inhibitor IκB, thereby allowing NF-κB to enter nuclei and rapidly induce coordinate sets of defense-related genes. NF-κB does this by binding to *cis*-acting κB sites in promoters and enhancers of these genes bearing the consensus sequence 5′-GGGPuNNPyPyCC-3′.

In this review we focus on recent developments in the field. For more complete perspectives on NF-κB, we refer the reader to previous reviews that include comprehensive and specifically focused treatments of the subject (Baeuerle 1991; Blank et al 1992; Bose 1992; Bours et al 1992a; Gilmore 1992; Israël 1992; Nolan & Baltimore 1992; Schreck et al 1992a; Beg & Baldwin 1993; Collins 1993; Gilmore & Morin 1993; Grilli et al 1993; Grimm & Baeuerle 1993; Hay 1993; Liou & Baltimore 1993; Kaltschmidt et al 1993; Muller et al 1993; Wasserman 1993; Baeuerle & Henkel 1994; Lenardo & Siebenlist 1994).

MOLECULAR STRUCTURE AND FUNCTION

The Family of Rel/NF-κB Proteins

The active, DNA-binding forms of NF-κB transcription factors are dimeric complexes, composed of various combinations of members of the Rel/NF-κB family of polypeptides. This family is distinguished by the presence of a so-called Rel homology domain (RHD) of about 300 amino acids in length, which displays ~ 35 to 61% identity between various family members (Bours et al 1992a,b; Ryseck et al 1992; Ip et al 1993). The RHD determines DNA binding to κB elements, dimerization with identical or other members of the family, as well as interaction with the IκB family of proteins (see below). The Rel/NF-κB family members can be grouped into two classes; one consists of the p105 and p100 precursor proteins, which are processed proteolytically to the mature p50 and p52 forms, respectively (Bours et al 1990; Ghosh et al 1990; Kieran et al 1990; Meyer et al 1991; Neri et al 1991; Schmid et al 1991; Bours et al 1992b; Mercurio et al 1992), and the other class consists of the Rel (c-Rel), v-Rel, RelA (p65) and RelB proteins (Stephens et al 1983; Wilhelmsen et al 1984; Brownell et al 1989; Grumont & Gerondakis 1989; Nolan et al 1991; Ruben et al 1991,1992; Ballard et al 1992; Ryseck et al 1992), as well as the *Drosophila* proteins dorsal and Dif (Steward 1987; Ip et al 1993) (see Table 1 for nomenclature). A critical distinguishing characteristic between the former (p50 and p52) and latter (p65, Rel, dorsal and Dif) classes is their discrepant ability to activate transcription. Members of the former class, if associated as homodimers, are weak or inert gene activators. In contrast, dimeric forms of NF-κB containing at least one subunit of the latter category function as potent activators of gene expression. In molecular terms, the activating members of the NF-κB family are understood to contain activation domains that lie outside the RHD.

Table 1 Rel/NF-κB and IκB proteins

Proteins	Other names	Genes	Chromosomal locus
p50 or p105 (NF-κB1)	p110, KBF1, EBP-1	*nfkb1*	NFKB1
p52 or p100 (NF-κB2)	p50B or p97, p49 or p100, p55 or p98, Lyt10, H2TF1	*nfkb2*	NFKB2
Rel	c-Rel	*rel*	REL
v-Rel	—	*v-rel*	—
RelA	p65	*rela*	RELA
RelB	I-Rel	*relb*	RELB
dorsal	—	*dorsal*	—
Dif, Cif	dorsal-related immunity factor cecropia immunoresponsive factor	*dif*	—
IκB-α	MAD-3, pp40, RL/IF-1, ECI-6	*ikba*	IKBA
IκB-β			
IκB-γ	p105/pdI, C-terminal portion of p105	*nfkb1*	NFKB1
Bcl-3		*bcl-3*	BCL3
cactus	—	*cactus*	—

It is unclear whether processing of the p105 or p100 precursor proteins into their mature forms is a regulated process. Evidence indicates that such processing is upregulated during stimulation with extracellular signals (Mellits et al 1993; Mercurio et al 1993). Other studies have indicated that processing is a relatively slow, constitutive process (Fan & Maniatis 1991; Baeuerle & Henkel 1994). p105 is processed at a significantly faster rate than p100 (Mercurio et al 1993; K Brown & U Siebenlist, unpublished observations). The reaction is ATP dependent (Fan & Maniatis 1991). The mature proteins end in a glycine-rich region located directly adjacent to the RHD. The glycine-rich region has been interpreted to function as a flexible hinge in the precursor, also providing potential access to cleavage by endoproteases. The resulting N-terminal halves of the p105 or p100 molecules constitute the mature p50 or p52 molecules, respectively, each containing the intact RHD. Separate, processed C-terminal parts have not been detected in cells, presumably because they are rapidly degraded, either as a consequence of a subsequent, independent processing step or as part of a progressive reaction initiated by the initial processing (Fan & Maniatis 1991; Rice et al 1992). In this regard, precursors with even short, incomplete C-terminal extensions beyond the RHD can be processed into the final mature size, which suggests that an intact C-terminus is not required for processing (Blank et al 1991; Fan & Maniatis 1991). The structure and function of the C-terminal domains removed from p105 and p100 during processing are discussed below.

NF-κB: A Family of Multiple Distinct Dimers

The members of the Rel/NF-κB family of proteins can form almost all theoretically possible homo- and heterodimers, generating many complexes through combinatorial mixing. Dimerization is an obligatory regimen for DNA binding (Logeat et al 1991; Bressler et al 1993), probably because both subunits interact with the κB DNA element (see below). κB sites are dyad symmetric, and each subunit of the NF-κB dimer interacts with a half site of the recognition sequence.

The p50/RelA (p50/p65) complex is present in essentially all cells and is usually the most abundant dimeric complex. Among the other possible dimeric complexes, homodimers of RelA or Rel or RelA/Rel heterodimers are usually detected at only very low levels, but such complexes may, nevertheless, play unique and significant roles in the context of specific promoters (Hansen et al 1992; Nakayama et al 1992; Bakalkin et al 1993; Costello et al 1993; Ganchi et al 1993; Kunsch & Rosen 1993). Complexes that have not been detected, either in vitro and/or in vivo, are RelB homodimers (Ryseck et al 1992; Dobrzanski et al 1993) and RelB/RelA and RelB/Rel heterodimers.

The various dimeric complexes differ in their preference for certain κB sites, transactivation potentials, kinetics of nuclear translocation, and levels of expression in tissue. The nearly ubiquitously expressed p50/RelA tends to be rapidly translocated to the nucleus in response to extracellular activating signals, whereas other complexes such as p50/Rel accumulate in nuclei more slowly, exhibiting a delay of several hours before reaching peak levels (Molitor et al 1990; Doerre et al 1993). The reason for this delay is not understood. Rel complexes have been proposed to function as late-acting competitive down-regulators of genes induced by RelA dimers at early times after stimulation (Ballard et al 1992; Doerre et al 1993; La Rosa et al 1994). However, the transfection data on which this conclusion is based may simply reflect a relatively lower (but still significant) transactivation potential of Rel in these cells. In other cells, including embryonal cells, Rel potently transactivated many κB-dependent reporter constructs (Inoue et al 1991; Richardson & Gilmore 1991; Ishikawa et al 1993; Inuzuka et al 1994).

Rel-containing complexes have been observed in many cell lines, but they appear to be preferentially present in hematopoietic cells, particularly in lymphoid cells (Brownell et al 1987, 1989; Feuillard et al 1994). This foreshadows a greater role for Rel in regulating gene expression in such cells, especially in B cells. RelB complexes may be even more restricted, with expression in tissue detectable primarily in interdigitating dendritic cells in thymus and probably in spleen, as judged by in situ hybridization (Carrasco et al 1993). RelB mRNA could not be detected during early mouse embryogenesis, but appears later, at times when the immune system matures and when dendritic cells begin to function to negatively and positively select T cells (Carrasco et al 1993). This correlation portends an important role for RelB in antigen presentation and T cell activation and selection. However, RelB is induced also in fibroblast cell lines subjected to serum stimulation (Ryseck et al 1992). In another study, RelB complexes have also been reported in some B cells (Lernbecher et al 1993). Together, these studies indicate that high expression of Rel and RelB is restricted to certain tissues but that lower and possibly significant levels may be present elsewhere.

Among the various members of the Rel/NF-κB family of proteins, p50 and p52 are most similar in primary amino acid sequence and behave almost identically in transfection experiments when paired with the transactivators RelA, Rel, or RelB, with which they heterodimerize (Bours et al 1992b; G Franzoso et al, unpublished observations). However, p50- and p52-containing complexes may differ functionally in the context of specific promoters where they interact with other transcription factors (Perkins et al 1992).

Protein Domains and their Functions

The highly conserved RHD is responsible for DNA binding, dimerization, nuclear translocation, and binding to IκB inhibitors, and it is the only region shared by all members of this family. Deletions and site-directed mutagenesis have located the DNA-binding region to the N-terminal part of this ~ 300 amino acid domain (Logeat et al 1991; Bressler et al 1993; Coleman et al 1993). A short stretch of amino acids at the beginning of the domain has been found essential for binding (the RXXRXRXXC motif) and is believed to contact DNA directly (Kumar et al 1992; Bressler et al 1993; Toledano et al 1993). The critical cysteine residue within this motif must be in a reduced state because its oxidation interferes with DNA binding (Toledano & Leonard 1991; Kumar et al 1992; Matthews et al 1992, 1993a; Hayashi et al 1993b; Toledano et al 1993). A second region ~ 100 amino acids further C-terminal to the RHD also contributes significantly to binding (Bressler et al 1993). Neither region obviously conforms with known DNA-binding motifs, which suggests that this family of proteins encodes a previously unrecognized DNA-binding motif (Liu et al 1994). Deletions or mutations in the p50 subunit, which interfered with DNA binding but still formed heterodimers with RelA, acted as dominant negative mutants in transfection experiments inhibiting transactivation by RelA (Logeat et al 1991; Bressler et al 1993).

Various studies have indicated that the C-terminal half of the RHD facilitates dimerization (Logeat et al 1991; Bressler et al 1993; Doerre et al 1993; Ganchi et al 1993). The structure of this region is also unknown. Dimerization with different partners may involve distinct amino acids. A potential cAMP-dependent protein kinase (PKA) phosphorylation site or an internal cysteine, when mutated in RelA, severely interferes with homodimerization, but not heterodimerization with p50 (Ganchi et al 1993). It is conceivable that phosphorylation of the PKA site could regulate dimer choices in RelA. The PKA site is conserved among the members of the Rel/NF-κB family, with the exception of RelB and p52 (Bours et al 1992b; Ryseck et al 1992).

The extreme C-terminal end of the RHD contains a conserved cluster of positively charged amino acids that function as a nuclear localization sequence (NLS) and that appear to be important for interaction with the inhibitory IκB proteins (see below) (Blank et al 1991; Beg et al 1992; Ganchi et al 1992; Henkel et al 1992; Matthews et al 1993b; Zabel et al 1993).

RelA (p65), Rel, RelB, and the *Drosophila* protein dorsal (and most likely Dif) contain demonstrable activation domains in polypeptide segments located on the carboxyl terminal side of the RHD (Bull et al 1990; Kamens et al 1990; Ip et al 1991; Richardson & Gilmore 1991; Schmitz & Baeuerle 1991; Ballard et al 1992; Dobrzanski et al 1993) (Figure 1 shows a schematic representation of all members of Rel/NF-κB/dorsal and IκB families). In the case of RelB, an additional activation function has been attributed to its unique N-terminal extension, a short region preceding the RHD that may encode a leucine-zipper-like motif (Dobrzanski et al 1993). According to one report, RelB (termed I-Rel) acted as a dominant inhibitor of NF-κB (Ruben et al 1992), but ample evidence now indicates that RelB functions to potently induce transcription (Bours et al 1992b, 1994; Ryseck et al 1992; Dobrzanski et al 1993). Rel contains two distinct regions that could participate in transactivation (Ishikawa et al 1993; Sarkar & Gilmore 1993). The most C-terminal of these two domains of Rel, as well as the potent transactivation domain present in RelA and the C-terminal domain of RelB, may all belong to the class of "acidic transactivators" (Bull et al 1990; Kamens et al 1990; Schmitz & Baeuerle 1991; Dobrzanski et al 1993), exemplified by the VP16 protein. V-Rel lacks this particular transactivating domain (Kamens et al 1990; Ishikawa et al 1993; Sarkar & Gilmore 1993), and this difference from Rel may be critical to oncogenic potential of the viral form (see below) (Capobianco et al 1990;

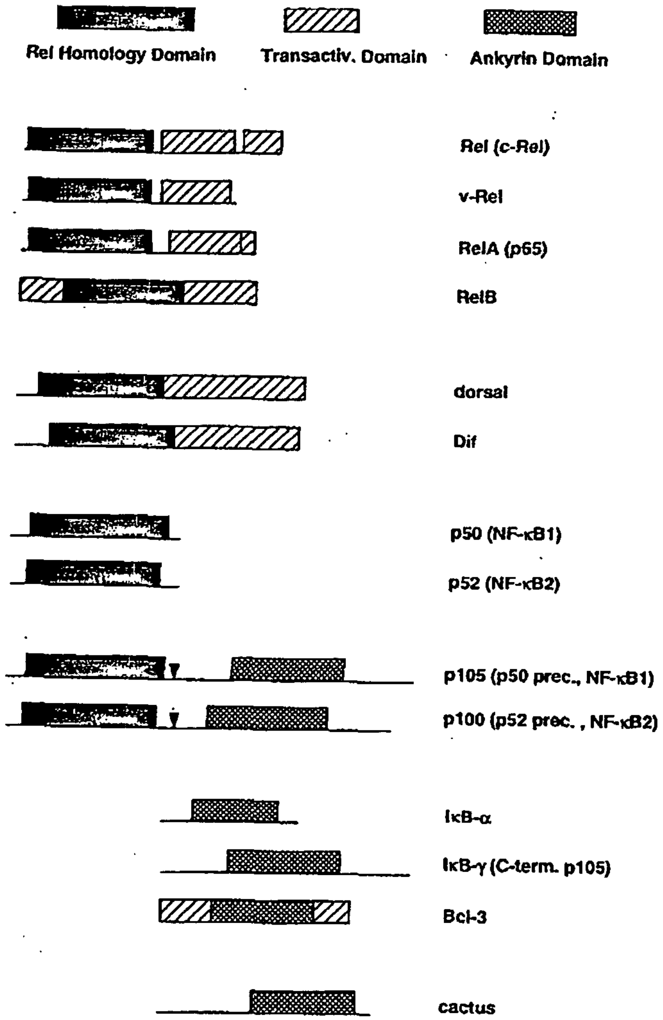

Figure 1 Schematic representation of the various members of the Rel/NF-κB/dorsal and the IκB family of polypeptides. Functional domains are indicated; in most cases the borders of the transactivation domains have not been precisely mapped. The putative transactivation domain of Dif has not been directly demonstrated. See text for details.

Kamens et al 1990; Mosialis et al 1991; Diehl et al 1993; Hrdlickova et al 1994a,b; Kralova et al 1994; Nehyba et al 1994). The residual transactivating function of v-Rel can be observed only in undifferentiated cells (Walker et al 1992; Ishikawa et al 1993; Inuzuka et al 1994; Hrdlickova et al 1994b), whereas Rel can transactivate in a wide range of cells (Kamens et al 1990; Inoue et al 1991; Ishikawa et al 1993). Transactivation by these factors is probably mediated by direct physical interaction with the basal transcription apparatus, possibly allowing the formation of a stable pre-initiation complex, but the precise mechanisms remain to be determined (see Kerr et al 1993; Xu et al 1993).

Different NF-κB dimers display different binding affinities with respect to certain κB sites (Urban & Baeuerle 1990; Neri et al 1991; Bours et al 1992b; Kunsch et al 1992; Perkins et al 1992; Franzoso et al 1993). Dissociation constants of a number of complexes at preferred binding sites were reported in the range of 10^{-12} M (Urban & Baeuerle 1990; Fujita et al 1992; Kretzschmar et al 1992), which indicates strong binding. The transactivation potential of a particular NF-κB dimer in vivo may depend not only on the affinity for a given κB element but also on the DNA sequence-dependent conformation adopted by the bound dimer. Different κB sites appear to allow different protein surfaces to be exposed, and transactivation potential does not necessarily correlate with binding affinity in vitro (Fujita et al 1992; Perkins et al 1992).

Most κB elements are *somewhat palindromic, but rarely completely so,* which gives these elements a directional sense relative to the transcriptional start site (Baeuerle 1991; Urban & Baeuerle 1990, 1991). This is likely to be important functionally because the p50 subunit of a p50/RelA heterodimer has been shown to interact preferentially with the 5′ half site of the prototypical immunoglobulin (Ig) κB site (5′-GGGAC), while the 3′ half site (5′-GGAAA, opposite strand), which is more variable among different κB sites, *interacts preferentially with the RelA (p65) subunit* (Urban & Baeuerle 1991; Kunsch et al 1992). Consequently, p50 homodimers prefer to bind palindromic sequences consisting of two Ig κB 5′ half sites, whereas RelA homodimers prefer duplicated Ig κB 3′ half sites. The three G residues in the 5′ half site (top strand) and the two G residues in the 3′ half site (bottom strand) of the IgκB site may be directly contacted, as judged by methylation protection and interference experiments (Baldwin & Sharp 1988; Baeuerle & Baltimore 1989; Israël et al 1989). The more variable bases in between, primarily AT base pairs, may be important for the bending of DNA observed upon binding (Schreck et al 1990). That DNA bending might be critically involved is further supported by work with the IFN-β promoter κB site, where the HMG-I/Y proteins, which are known to facilitate DNA bending, also enhance binding of p50/RelA. The HMG-I/Y proteins recognize the particular AT base pairs of the IFN-β κB site in the minor groove and bind simultaneously with p50/RelA (Thanos & Maniatis 1992).

The Family of IκB-Related Proteins

The IκB family of proteins includes IκB-α, the precursor proteins p105 and p100, IκB-γ, Bcl-3, and the *Drosophila* protein cactus. All members of the family share a partially conserved domain that harbors between six to eight ankyrin motifs, each 33-amino acids in length (Schmitz et al 1991; Blank et al 1992; Nolan & Baltimore 1992). That these motifs are regulators of NF-κB activity was first recognized with the cloning of the p105 precursor (Bours et al 1990; Kieran et al 1990), where they were discovered to inhibit DNA binding of the N-terminal Rel/NF-κB homology domain (Bours et al 1990; Ghosh et al 1990; Kieran et al 1990). The members of this protein family interfere with nuclear translocation and DNA binding of Rel/NF-κB complexes, with several notable exceptions. Although IκB proteins generally interact with dimers (Beg et al 1992), evidence indicates binding of IκB-α to RelA monomers as well (Ganchi et al 1993; P Bressler & U Siebenlist, unpublished observations).

IκB-α (and the ankyrin domain of the p105 precursor) interact with and shield the nuclear localization signal (NLS) located at the end of the RHD domain (Beg et al 1992; Ganchi et al 1992; Henkel et al 1992; Matthews et al 1993b; Zabel et al 1993). IκB-α does so as a monomer with all dimeric NF-κB complexes tested, which suggests that it shields the positively charged NLSs of both subunits of these dimers (Hatada et al 1993). A short, negatively charged region of IκB-α located adjacent to the most C-terminal (sixth) ankyrin motif may be critical (Hatada et al 1992, 1993). An analogous acidic region in the p105 precursor located between the last two ankyrin motifs appears to be necessary for the inhibitory activity of that protein (Blank et al 1991; Hatada et al 1993). In addition, cytoplasmic retention may be assisted by a cooperating domain at the C-termini of Rel and dorsal (Hannink & Temin 1989; Capobianco et al 1990; Isoda et al 1992; Norris & Manley 1992; Diehl et al 1993). Although these regions do not appear to be necessary for binding by IκB-α (see below), they may nevertheless cooperate with IκB-α to help cytoplasmic retention. In the case of RelA, a C-terminal domain was also reported to assist in IκB-α-mediated inhibition of DNA binding (Ganchi et al 1992). Homodimers of p50 or p52 are less efficiently retained in the cytoplasm (Franzoso et al 1992; Kang et al 1992), although they bind IκB-α (Beg et al 1992; Ganchi et al 1992; Franzoso et al 1993). The interaction with the homodimers may not be physiologically relevant. However, IκB-α binds tightly and functionally inhibits all dimeric complexes containing any of the transactivating subunits (RelA, Rel, RelB), in particular those containing RelA.

IκB-α not only prevents DNA binding of the strongly transactivating complexes, but it also dissociates prebound complexes from their cognate DNA sites (Zabel & Baeuerle 1990). While complexes containing either RelA, Rel, or RelB subunits are inhibited in this way (Baeuerle & Baltimore 1988a; Haskill et al 1991; Beg et al 1992; Davis et al 1991; Inoue et al 1992b; Tewari et al 1992), DNA binding by p50 and p52 homodimers is essentially unaffected by IκB-α (Beg et al 1992; Kerr et al 1992; Franzoso et al 1993; Nolan et al 1993). IκB-α is apparently readily displaced from these homodimers in the presence of a κB binding site (Beg et al 1992). To date, no consensus has emerged as to how DNA binding of dimers containing RelA, Rel, or RelB is blocked by IκB. According to one report, IκB-α binding to the N-terminal DNA-binding region of Rel is both necessary and sufficient (Kerr et al 1991), whereas other reports suggest some binding to the N-terminal and a possibly stronger interaction with the NLS (Diehl et al 1993; Kumar & Gelinas 1993). In addition, IκB-α binding to RelA has been localized to the dimerization domain, which also includes the adjoining NLS (Ganchi et al 1992, 1993). According to the model derived from the latter data, DNA binding is blocked indirectly, possibly by changing the interaction between dimer subunits. This mode of action would enable IκB-α to dissociate stably bound complexes from DNA without requiring direct access to the DNA-contacting portion of the bound protein.

Initial purification procedures for IκB activity revealed two distinct fractions termed IκB-α and IκB-β (Zabel & Baeuerle 1990; Link et al 1992). Despite earlier confusion in the literature, direct evidence now demonstrates IκB-α function to be encoded by the cloned human MAD-3 (Haskill et al 1991), chicken pp40 (Davis et al 1991), rat RL/IF-1 (Tewari et al 1992), and porcine ECI-6 (de Martin et al 1993) proteins (Zabel et al 1993). The identity of IκB-β remains unresolved. Based on their charge differences (Link et al 1992), the absence of *immunological cross-reactivity* (Zabel et al 1993), and difference in molecular weights, (IκB-α is 37 K and IκB-β is reportedly 43 K (Zabel & Baeuerle 1990). IκB-β may be encoded by a separate gene from IκB-α. Also, IκB-β activity is inactivated by phosphatase treatment in vitro, in distinction from the IκB-α-containing fraction (Link et al 1992).

Bcl-3 is an IκB-α-related protein that was first discovered as a translocation of its gene into the immunoglobulin locus in a subset of chronic lymphocytic leukemias (Ohno et al 1990). Whereas IκB-α preferentially interacts with the transactivating complexes, especially those containing RelA, Bcl-3 primarily targets homodimers of p50 and p52 (Franzoso et al 1992, 1993; Hatada et al 1992; Wulczyn et al 1992; Bours et al 1993; Fujita et al 1993; Naumann et al 1993; Nolan et al 1993). Also, Bcl-3 does not interfere with the nuclear translocation of these complexes. Indeed, Bcl-3 can be readily detected in cell nuclei (Bours et al 1993; Franzoso et al 1993; Nolan et al 1993), although not in all cells (Naumann et al 1993). These differences between IκB-α and Bcl-3 (Hatada et al 1992; Nolan & Baltimore 1992), together with unique features of Bcl-3 encoded outside of the ankyrin domain (Bours et al 1993), indicate a very different function of this protein. Instead of inhibiting transactivation by NF-κB, Bcl-3 promotes κB-dependent transcription (see below). Using in vitro cross-linking experiments, up to two Bcl-3 monomers were reportedly able to interact with a single p50 homodimer (Wulczyn et al 1992). This contrasts with the interaction of only one IκB-α monomer with a p50/RelA dimer (Hatada et al 1993).

The p105 and p100 precursor proteins function as IκB-like proteins (Blank et al 1991; Henkel et al 1992; Rice et al 1992, Mercurio et al 1993; Naumann et al 1993; Scheinman et al 1993; Beraud et al 1994). p105 and p100 are able to dimerize with the various Rel proteins, and they inhibit these proteins from translocating to the nucleus, even in the absence of IκB-α (Henkel et al 1992; Rice et al 1992; Mercurio et al 1993; Naumann et al 1993). Given the relatively long survival of the precursor forms prior to processing, they may function as physiological inhibitors of NF-κB activity. At least part of the pool of Rel proteins is found in association with the p105 and p100 precursors, to the exclusion of IκB-α (Rice et al 1992).

The precursor is speculated possibly to have an IκB-like regulated activity (Mercurio et al 1993). Precursor-containing cytoplasmic complexes not associated with IκB-α could represent an alternative source for nuclear NF-κB activity, which may be activated to translocate to the nucleus by direct signal-induced proteolytic processing. However, no current evidence exists that demonstrates a rapid signal-dependent release from precursor inhibition and subsequent nuclear translocation, although a moderate increase in processing has been reported after activation of some cells (Mercurio et al 1993). The precursor-associated cytoplasmic complexes may not represent a reservoir poised for direct immediate activation but, rather, they may primarily serve as a reservoir to replenish the signal-responsive IκB-α-associated complexes.

IκB-γ contains the C-terminal half of the p105 precursor molecule. It is not a product of processing but rather is synthesized from a separately initiated mRNA present in certain mouse B cells (Inoue et al 1992a; Liou et al 1992), where IκB-γ protein is also found. Nothing is known about control of expression of this mRNA in mouse B cells. Although this protein is capable of inhibiting various NF-κB complexes in vitro in a manner somewhat similar to that of IκB-α (Inoue et al 1992a; Liou et al 1992), its physiological relevance is unclear and no equivalent mRNA or protein has been detected in any human B cells.

The family of IκB proteins is part of a larger family of proteins with more divergent ankyrin motifs (Schmitz et al 1991; Blank et al 1992; Hatada et al 1992; Michaely & Bennett 1992; Nolan & Baltimore 1992). No obvious consensus of biological function exists within the larger family of proteins with ankyrin motifs. The human ankyrin protein, and, in particular its ankyrin domain, links ion channels to cytoskeletal structures (Lux et al 1990). Several transcription factors, including two yeast cell cycle proteins, contain ankyrin repeats, as do various developmentally important regulators in *Drosophila*, nematodes, and frogs (Michaely & Bennett 1992). Most likely, the common denominator between these and other ankyrin motif-containing proteins is that the motifs define structures for protein-protein interactions. The more highly conserved segments within each ankyrin motif are likely to form amphipathic α-helices [although the existence of β-sheets has been hypothesized as well (Michaely & Bennett 1992)], whereas the more divergent segments of the repeats may represent loops that also determine binding specificity. Together the individual ankyrin motifs may form a tertiary structure, possibly by bundling of α-helices (Gay & Ntwasa 1993). For IκB-α, Bcl 3, and p105, the whole ankyrin domain is necessary for inhibitory activity. In the case of IκB-α and p105, a short, negatively charged segment is also required for full inhibition (Hatada et al 1992, 1993; Wulczyn et al 1992; Bours et al 1993, Franzoso et al 1993; see also Blank et al 1991; Inoue et al 1992b).

Historical Perspectives and Nomenclature

NF-κB DNA binding, and transactivation activity was first detected with the κB element of the immunoglobulin enhancer in certain murine B cells (Sen & Baltimore 1986a,b). This discovery was born of prior work that mapped an inducible DNAaseI hypersensitive site to this region in response to differentiation of a mouse preB cell (Chung et al 1983; Parslow & Granner 1983). This κB element was shown to be critical to B cell-specific expression of enhancer function, which led to the conclusion that the bound factor, called NF-κB, was a lineage and differentiation stage-specific factor for B cells (Sen & Baltimore 1986a). It was soon recognized, however, that this binding activity was ubiquitous and could be detected in many cells but usually only after stimulation of such cells by various agents (see Lenardo & Baltimore 1989). Subsequently, the IκB inhibitor was identified as a protein capable of retaining NF-κB in the cytoplasm in the absence of activating signals (Baeuerle & Baltimore 1988a,b). This inhibitor could be dissociated in vitro with detergents (deoxycholate; DOC), which liberated NF-κB (Baeuerle & Baltimore 1988b).

Prior to cloning of the genes, the various NF-κB activities could not be distinguished by the standard electrophoretic mobility shift assay, but it is now known that several different dimeric complexes account for what was earlier thought to be a single NF-κB complex. To be consistent with prior literature, we use the term NF-κB or Rel/NF-κB to collectively denote all κB-binding activity of this family of proteins, and we refer to specific dimers by naming their subunits.

The first cloning of a gene (p50/p105) encoding a component of the NF-κB binding activity was the result of independent and simultaneous efforts in several laboratories (Bours et al 1990; Ghosh et al 1990; Kieran et al 1990; Meyer et al 1991). This development dramatically impacted the field since it soon led to the discovery (and cloning) of a family of Rel/NF-κB and IκB proteins. In addition, it merged this field of research with that of the previously discovered proteins Rel/v-Rel and dorsal.

Table 1 indicates the nomenclature proposed for the individual members of the Rel/NF-κB and IκB families (adapted from a consensus reached in October 1992 at a meeting entitled *NF-κB, Rel and Dorsal: Structure and Function,* held at the Howard Hughes Medical Institute in Bethesda, MD). For simplicity and clarity, we use only the names p50, p52 (or their precursors p105, p100, respectively), RelA, Rel, v-Rel, and RelB throughout this review.

DIRECT MECHANISMS FOR ACTIVATION

Modification and Degradation of IκB-α

Activation of NF-κB from cytoplasmic pools correlates temporally with proteolytic degradation of the inhibitory IκB-α protein. Nearly complete degradation of the inhibitor occurs within minutes after administration of extracellular stimuli such as TNF-α or PMA plus ionomycin, an observation that has now been confirmed in many laboratories (Beg et al 1993; Brown et al 1993; Sun et al 1993, see also Cordle et al 1993; de Martin et al 1993; Henkel et al 1993; Mellits et al 1993; Rice & Ernst 1993; Scott et al 1993; Chiao et al 1994; Read et al 1994). In addition, a post-translational phosphorylation of IκB-α accompanies the degradation (Beg et al 1993; Brown et al 1993; Cordle et al 1993; Sun et al 1994). The modified form of IκB-α does not accumulate but is detected (as a protein with slightly slower electrophoretic mobility) only transiently shortly after stimulation of cells. This is consistent with the idea that phosphorylation tags IκB-α for rapid degradation, although this is still unproven. As IκB-α disappears from cells, NF-κB is freed to enter nuclei and induce genes (Brown et al 1993; Sun et al 1993).

A different model for activation is based on the observation that free, uncomplexed IκB-α is extremely unstable in cells, while complexed IκB is more stable (Beg et al 1993; Brown et al 1993; Rice & Ernst 1993; Scott et al 1993; Sun et al 1993). According to this model (see discussion in Beg et al 1993), phosphorylation of IκB-α releases the inhibitor from NF-κB, thus leaving it unprotected from constitutively active proteases, which then digest the free form. Therefore, proteolytic degradation per se would not be an integral part of activation. This view is also based on early reports indicating that several kinases activate IκB-bound NF-κB in vitro and phosphorylate and inactivate partially purified IκB (Shirakawa & Mizel 1989; Ghosh & Baltimore 1990).

Recently we obtained evidence in support of an essential role for degradation in activating NF-κB, contrary to the above model, which does not envision proteolytic degradation as a critical activating event. IκB-α protein, which is modified by phosphorylation in response to cellular signals, in fact, remains associated with NF-κB, as demonstrated by co-immunoprecipitation with anti-RelA antibodies (YC Lin & U Siebenlist, unpublished observations). This favors the view that proteases are required to liberate NF-κB activity, presumably acting on the NF-κB-IκB-α complex in situ.

Evidence for a critical role of proteases also emerges from studies with inhibitors of chymotrypsin-like proteases, inhibitors that appear to specifically block degradation of IκB-α and activation of NF-κB (Henkel et al 1993; Mellits et al 1993; Chiao et al 1994). However, the interpretation of these data is complicated by the discovery that these protease inhibitors also block the signal-dependent modification of IκB-α (YC Lin et al, unpublished observations). It is therefore unclear how these protease inhibitors function to inhibit activation of NF-κB, but they may have unappreciated activities, including potential effects on signaling kinases.

According to the simplest form of the current model, signal-dependent phosphorylation of IκB-α is sufficient to trigger proteolytic degradation. However, a separate signal-dependent induction of protease activity has not been ruled out. To date the protease(s) involved has not been identified. Intermediate fragments resulting from the degradation of IκB-α have not been reported, which possibly reflects a rapid and complete proteolysis.

The kinase directly responsible for mediating signal-dependent phosphorylation has not been definitively identified either, although some evidence implicates Raf as this kinase. This interpretation is based largely on indirect experiments. For example, cells transfected with active forms of Raf show constitutively active NF-κB. Likewise, cells that express dominant negative forms of Raf fail to activate NF-κB upon exposure to TNF-α, serum, PMA, or UV irradiation (Bruder et al 1993; Devary et al 1993; Finco & Baldwin 1993; Li & Sedivy 1993). These data argue for a role of Raf at some stage during stimulation by these agents (see below). That Raf may be an IκB-α kinase has also been suggested by a presumed direct physical interaction between Raf and IκB-α, as concluded from a yeast two-hybrid analysis in which genes for both proteins were specifically introduced (Li & Sedivy 1993). Furthermore, IκB-α can be phosphorylated by Raf in vitro, although this by itself does not establish IκB-α as a physiological target (Li & Sedivy 1993).

Modification of NF-κB

Although IκB-α appears to be the primary target for activation signals, the associated NF-κB proteins may also be important targets. dorsal is reported to be rapidly phosphorylated by protein kinase A (PKA) in response to activation

(Norris & Manley 1992), and Rel and p105 become tyrosine phosphorylated (Neumann et al 1992; see also Mellits et al 1993). Furthermore, an as yet unidentified kinase has been purified in association with RelA (Hayashi et al 1993a). Despite these observations, there is at present no convincing evidence to indicate a role for modification of NF-κB subunits in triggering activation. In fact, activated nuclear NF-κB, when extracted from cells, remains sensitive to IκB-α inhibition in vitro (Rice & Ernst 1993). Modifications could result following dissociation of IκB-α whereupon new protein surfaces become exposed on NF-κB dimers (e.g. the PKA site located adjacent to the NLS, see above). Likewise, translocation of NF-κB into the nucleus could well result in exposure to new protein kinases. Phosphorylation of NF-κB subunits, if relevant, could affect functions such as transcriptional activation.

Cactus and Dorsal

Considerable genetic data exist concerning the activation of the *Drosophila* protein dorsal during embryogenesis (reviewed by Govind & Steward 1991; St. Johnston & Nüsslein-Volhard 1992). Dorsal is kept in the cytoplasm by cactus, the *Drosophila* homologue of IκB-α, during the early developmental stages. After ten nuclear division cycles, the embryo receives a positional cue through the toll receptor on the ventral side only. In response, dorsal enters nuclei in a gradient fashion. In addition to the negative role played by cactus, which is relieved by the toll-mediated activating signal, at least two further proteins are involved in the signaling cascade linking toll to dorsal, namely tube and pelle. Whereas little is known about the function of tube, pelle is a serine protein kinase that may be a direct regulator of cactus (Letson et al 1991; Shelton & Wasserman 1993). pelle is distantly related to Raf and many other kinases. Another possible parallel between *Drosophila* and mammalian regulation is suggested by the primary amino acid sequence similarity between the cytoplasmic tails of the toll and the mammalian IL-1 receptor (Schneider et al 1991; Wasserman 1993); IL-1 is a potent activator of NF-κB. Since this signaling pathway may have been conserved through evolution, pelle and tube may have direct mammalian counterparts.

SIGNALS AND SIGNALING PATHWAYS FOR NF-κB ACTIVATION

Table 2 lists signals that lead to activation of NF-κB in some cells. Although very different, most signals are directly or indirectly associated with a pathogenic event or physical stress. All signals so far tested target IκB-α, as demonstrated by its ensuing degradation. On the one hand, it is possible that all of these divergent signals converge on an effector molecule upstream of IκB-α,

Table 2 NF-κB-activating stimuli

Cytokines	Tumor necrosis factor-α (TNF-α)
	Lymphotoxin (LT) (TNF-β)
	Interleukin-1 α and β (IL-1 α and β)
	Interleukin-2 (IL-2)
	Leukemia inhibitory factor (LIF)
	(Interferon-γ)
	(Macrophage colony-stimulating factor) (M-CSF)
	(Granulocyte/macrophage colony-stimulating factor) (GM-CSF)
Mitogens	Antigen
	Allogeneic stimulation
	Lectins (PHA, Con A)
	anti-αβ T cell receptor
	anti-CD3
	anti-CD2
	anti-CD28[1]
	Phorbol esters
	(Diacylglycerol) (DAG)
	Calcium ionophores (ionomycin, A2837)[1]
	anti-surface IgM
	(p39) (CD-40 ligand)
	Serum
	(Platelet-derived growth factor) (PDGF)
Other biological mediators	Leukotriene B4
	(Prostaglandin E2) (PGE2)
	(Insulin)
Bacteria and bacterial products	*Shigella flexneri*
	Mycobacterium tuberculosis
	Cell wall products:
	Lipopolysaccharide (LPS)
	Muramyl peptides
	(G(Anh)MTetra)
	Toxins:
	Staphylococcus enterotoxin A and B (SEA and SEB)
	Toxic shock syndrome toxin-1 (TSST-1)
	(Cholera toxin)
Viruses and viral products	Human T cell leukemia virus-1 (HTLV-1)
	Tax
	Hepatitis B virus (HBV)
	Hbx
	MHBs[1]
	Epstein-Barr virus (EBV)
	EBNA-2
	LMP

Table 2 *(Continued)*

	Cytomegalovirus (CMV)
	(Human immunodeficiency virus-1) (HIV-1)
	Herpes simplex virus-1 (HSV-1)
	Human herpes virus-6 (HHV-6)
	Newcastle disease virus
	Sendai virus
	Adenovirus 5
	ds RNA
Eukaryotic parasite	*Theileria parva*
Physical stress	UV light
	Ionizing radiations (X and γ)
	(Photofrin plus red light)
	(Hypoxia)
	Partial hepatectomy
Oxidative stress	Hydrogen peroxide
	Butyl peroxide
	Oxidized lipids
	(Antimycin A)[1]
Chemical agents	Calyculin A
	Okadaic acid
	(Pervanadate)
	(Ceramide)
	(Dibutyrl c-AMP)
	(Forskolin)
	Protein synthesis inhibitors
	Cycloheximide
	Anisomycin
	Emetine

[1] These agents have been shown to active NF-κB in conbination with other stimuli for references see text and recent reviews (Baeuerle 1991; Grilli et al 1993). *Not cited* in these reviews are *S. flexneri* (Dyer et al 1993); muramyl peptides (Schreck et al 1992b); G(Anh)MTetra (Dokter et al 1994); SEA, SEB and TSST-1 (Trede et al 1993); cholera toxin and dibutyryl c-AMP (Muroi & Suzuki 1993; Parhami et al 1993); IL-2 (Brach et al 1992b); LIF (Gruss et al 1992); interferon-γ (Narumi et al 1992; Yasumoto et al 1992; Ohmori & *Hamilton 1993); PGE2* and forskolin (Muroi & Suzuki 1993); leukotriene B4 (Brach et al 1992a); insulin (Dominguez et al 1993); allogeneic stimulation (Jamieson et al 1991); p39 (Lalmanach-Girard et al 1993); PDGF (Olashaw et al 1992); X-rays (Brach et al 1993a); photofrin and red light (Ryter & Gomer 1993); pervanadate (Schieven et al 1993; Imbert et al 1994); partial *hepatectomy* (Tewari et al 1992); antimycin (Schulze-Osthoff et al 1993); emetine (Ghersa et al 1992). The activating stimuli listed in parentheses are less well characterized.

for example, an IκB-α kinase. Alternatively, IκB-α itself may be the common target of multiple independent pathways.

It is unclear whether all NF-κB activity that is released into the nucleus originates from a cytoplasmic pool that is bound to and inhibited by IκB-α. As discussed above, there appears to be at least one other pool of cytoplasmic complexes containing of precursor molecules p105 and p100. Despite almost total *degradation of IκB-α in response to potent stimuli, a major portion of NF-κB* protein usually remains in the cytoplasm (Sun et al 1994), presumably on account of the precursors. In addition, the putative distinct IκB-β protein could contribute to cytoplasmic retention as well. Despite such alternative cytoplasmic, IκB-α-independent pools, there is as yet no evidence for a direct, extracellular signal-responsive translocation of NF-κB complexes from such pools into nuclei.

Activation by TNF-α

Consistent with a role of NF-κB activity in a defensive response, the pro-inflammatory cytokines TNF-α, TNF-β, and IL-1 are potent activators of this transcription factor in a wide variety of cell types (Osborn et al 1989; Messer et al 1990; Hohmann et al 1990a; 1990b). TNF-α, one of the most potent effectors, signals through the 55 kd TNF-I receptor. Although the immediate receptor proximal events are unknown, a phosphatidylcholine-specific phospholipase C is activated to generate 1,2-diacylglycerol (DAG) in response to ligand (Dressler et al 1992; Schutze et al 1992). DAG, in turn, may initiate a signaling cascade that involves, in order, the activation of acidic sphingomyelinases—located in endosomal and lysosomal compartments—the subsequent generation of ceramide from sphingomyelin (Schutze et al 1992), and the ceramide-dependent activation of a serine/threonine kinase (Mathias et al 1991; Joseph et al 1993). In support of this pathway for TNF-α, NF-κB has been activated in permeabilized cells by exogenously added phospholipase C, DAG analogues, and purified acidic sphingomyelinase or ceramide (Schutze et al 1992). Neutral plasma membrane-associated sphingomyelinases have also been proposed to be involved in NF-κB activation, independent of DAG (Yang et al 1993). Addition of sphingomyelinase or cell-permeable ceramide analogues to intact cells induces NF-κB activity (Yang et al 1993).

Raf may lie on this pathway as well, since mitogen-activated protein (MAP) kinase kinase, a downstream effector of Raf function, is activated in response to TNF (Saklatvala et al 1993). Dominant negative Ras and Raf mutants block TNF-α-mediated activation of NF-κB (Devary et al 1993; Finco & Baldwin 1993). Mitochondria could present another downstream effector of ceramide because inhibitors of mitochondrial electron transport function impair TNF-induced activation of NF-κB (Schulze-Osthoff et al 1992). Likewise, depletion of this organelle had similar effects (Schulze-Osthoff et al 1993). As discussed below, mitochondria-generated reactive oxygen intermediates may be critical for activation. Other studies suggest an essential role for protein kinase C (PKC) zeta in TNF-α signaling to NF-κB, possibly as a component of the sphingomyelin pathway. A dominant negative PKC zeta protein inhibits NF-κB activation by TNF-α. Moreover, constitutively active PKC zeta activates NF-κB in NIH3T3 cells (Diaz-Meco et al 1993). Despite the production of DAG (which activates PKC), the activation of NF-κB by TNF-α appears to be independent of PKC. For example, inhibitors of PKC, which block PMA-induced activation of NF-κB, nevertheless fail to block TNF-α-induced activation (Meichle et al 1990).

IL-1 has been reported to signal via production of ceramide as well and it may feed into a pathway similar to that of TNF-α, as evidenced by the largely overlapping biological activities of the two cytokines (Mathias et al 1993). Herbimycin A, an inhibitor of tyrosine phosphorylation, interfers with IL-1α-induced NF-κB activation in T and B cells, which suggests a role for tyrosine kinases during early phases of signaling by this cytokine (Iwasaki et al 1992). Tyrosine kinases are also implicated in signaling by UV, gamma-irradiation, and bacterial lipopolysaccharide (LPS) (Devary et al 1993; Geng et al 1993; Schieven et al 1993).

Redox Regulation

Given the convergence of all signaling paths at or before the IκB-α target, it is possible that a common upstream effector exists, although no such molecule has been identified. However, several indirect lines of evidence suggest a role for reactive oxygen intermediates (ROI) as a common and critical denominator for various activating signals (Schreck et al 1992a,d). This conclusion is based largely on the inhibition of NF-κB activation by a variety of antioxidants. These reagents have been reported to block NF-κB activation in many instances, although the extent of this block appears to vary depending on cell and signal. Inhibitory antioxidants with diverse structural properties include N-acetyl-L-cysteine (NAC, a precursor of glutathione), dithiocarbamates, vitamin E derivatives, and various metal chelators.

Pyrolidinedithiocarbamate (PDTC) in particular has been widely used to inhibit the induced degradation of IκB-α, as shown with TNF-α and PMA stimulation of some Jurkat T cell lines (Beg et al 1993; Henkel et al 1993; Sun et al 1993). Activation of NF-κB by UV light is suppressed by NAC (Devary et al 1993). Support for the involvement of reactive oxygen intermediates as common messenger also derives from evidence showing elevated cellular levels for ROIs in response to TNF-α, IL-1; PMA, LPS, UV light, and gamma-irradiation (Schreck et al 1992a,d; Meyer et al 1993b, Geng et al 1993, Schieven et al 1993). Normal respiration produces basal levels of such intermediates. In the case of TNF-α, mitochondria may be involved in the signal-induced increase of such ROIs, as discussed above (Schulze-Osthoff et al 1992, 1993). Despite these observations, a direct functional role for ROIs in signaling to NF-κB remains to be proven.

Among various ROIs administered to cells in culture, such as hydrogen peroxide, hydroxyl radicals, superoxide, and singlet oxygen, only hydrogen peroxide is an effective activator of NF-κB (Schreck et al 1991, 1992a). It is possible that peroxides may specifically function in the NF-κB signaling cascade. Curiously, tyrosine kinase inhibitors block NF-κB activation following gamma-irradiation, a stimulant that is thought to work primarily through the immediate generation of ROI (Schieven et al 1993). Such inhibitors do not commonly block all NF-κB activation, which suggests that tyrosine kinase functions prior to the generation of ROIs.

Mitogenic Stimuli

Mitogenic stimuli for T and B cells activate NF-κB. In serum-deprived fibroblast cultures, NF-κB is transiently activated by serum stimulation, coincidently with a G_0-G_1 transition (Baldwin et al 1991). NF-κB is not activated in proliferating fibroblasts but may play a role in the initiation of the cell cycle from a resting state. Partial liver hepatectomy results in rapid tissue regeneration associated with immediate activation of NF-κB and the induction of many early response genes seen with other mitogenic systems (Tewari et al 1992). These cases indicate a possible link between NF-κB activation and cell proliferation.

Another connection between NF-κB function and growth is established through the induction of genes encoding growth factors and their receptors, such as IL-2, the IL-2 receptor-α chain, other growth-promoting cytokines, and the c-myc oncogene and IRF-1 and IRF-2 (Baldwin et al 1991; Duyao et al 1990, 1992; Kessler et al 1992; Harada et al 1994) (Table 3). Finally, NF-κB genes can become oncogenic upon genetic changes in structure and/or expression.

Activation by Viruses

The list of activators of NF-κB in Table 2 includes a growing number of viruses and viral products. Such activation is presumably beneficial to their lytic growth cycles; viruses may require NF-κB for transcription of their own genes, or they may require it to regulate various cellular genes. Induction of cellular growth-response genes may be critical to viral integration and replication functions. Viruses may activate NF-κB directly as part of the infection process through virion-associated molecules, or they may do so through viral products synthesized after infection.

Infection of T cells by HTLV I is known to immortalize these cells, a prelude to adult T cell leukemia (reviewed in Smith & Greene 1991). Transformation is mediated by the HTLV I Tax product (Nerenberg et al 1987; Grassman et al 1989), which activates a number of cellular transcription factors, among them NF-κB (Kelly et al 1992). Tax-mediated activation of NF-κB, in turn, contributes to the induced expression of many mitogen-response proteins (Kelly et al 1992) including the IL-2 growth factor and IL-2 receptor-α (Hoyos et al 1989; see also Smith & Greene 1991). The expression of Tax as a

Table 3 NF-γB-responsive genes

Cytokines and growth factors	Interleukin-1β
	Tumor necrosis factor α (TNF-α)
	Lymphotoxin (LT) (TNF-β)
	Interleukin-8
	IP-10
	Gro-α, -β and -γ
	(MIP-1α)
	(MCP-1/JE)
	(RANTES)
	Interleukin-2
	Macrophage colony-stimulating factor (M-CSF)
	Granulocyte/macrophage colony-stimulating factor (GM-CSF)
	Granulocyte colony-stimulating factor (G-CSF)
	(Erythropoietin)
	Interferon-β
	(Interferon-γ)
	Interleukin-6
	Proenkephalin
	(Transforming growth factor-β2) (TGF-β2)
Immunoreceptors	Immunoglobulin κ light chain (Ig-κ-LC)
	T cell receptor β chain
	Major histocompatibility complex class I (MHC-I)
	(Major histocompatibility complex II) (MHC-II)
	β2-microglobulin
	MHC-II invariant chain
	Tissue factor-1
	Interleukin-2 receptor α chain
	(CD7)
Adhesion molecules	Endothelial-leukocyte adhesion molecule-1 (ELAM-1)
	Vascular cell adhesion molecule-1 (VCAM-1)
	(Intracellular cell adhesion molecule-1) (ICAM-1)
Acute phase proteins	Angiotensinogen
	Serum amyloid A precursor
	Complement factor B
	Complement factor C4
	(Urokinase-type plasminogen activator)
Viruses	Human immunodeficiency virus 1 (HIV-1)
	(Human immunodeficiency virus 2) (HIV-2)
	Simian immunodeficiency virus *(macaques)* (SIVmac)
	Cytomegalovirus (CMV)
	Adenovirus
	Herpes simplex virus 1 (HSV-1)
	(Human neurotropic virus) (JCV)
	(Simian virus 40) (SV-40)
Transcription factors and regulators	Rel
	p105
	IκB-α
	Myc
	Interferon regulatory factor 1 (IRF-1)
	(Interferon regulatory factor 2) (IRF-2)
	A-20
Others	NO-synthetase
	(Apolipoprotein CIII)
	(Perforin)
	Vimentin
	(Decorin)

For references see text or recent reviews (Baeuerle 1991; Grilli et al 1993). Not cited in these reviews are: HSV-1 (Rong et al 1992); JCV (Ranganathan & Khalili et al 1993); tissue factor-1 (Mackman et al 1991); IP-10 (Ohmori & Hamilton 1993); erythropoietin (Lee-Huang et al 1993); A-20 (Krikos et al 1992); decorin (Santra et al 1994); MCP-1/JE (Rosi et al 1994); M-CSF (Brach et al 1993a; Sater et al 1994); interferon-γ (Sica et al 1992). *A definitive involvement of NF-κB in the regulation of the genes listed in parentheses* remains to be demonstrated.

transgene in mice caused tumors that regressed when treated with antisense RelA oligonucleotides, which implies a critical growth-associated role for the RelA subunit in these Tax-transformed tumor cells (Kitajima et al 1993).

Several possible mechanisms have been proposed for Tax activation of NF-κB including (*a*) the transcriptional induction of component parts of NF-κB by Tax (Arima et al 1991), (*b*) a post-translational mechanism (Lindholm et al 1992), possibly via generation of ROIs (Schreck et al 1992c), and (*c*) nuclear translocation of NF-κB and transactivation mediated by the reported physical interaction of Tax with the p50/p105 proteins (Hirai et al 1992; Suzuki et al 1993; Watanabe et al 1993). Recent discoveries indicate two potential ways in which Tax may activate NF-κB (T Kanno & U Siebenlist, unpublished observations). Tax was demonstrated to increase proteolytic degradation of the IκB-α protein, although by what means remains to be shown. Despite the continual, loss of IκB-α, high steady state levels of the inhibitor are maintained by NF-κB-induced new synthesis. Increased turnover of IκB-α may interfere with its inhibitory function, thus allowing some NF-κB to escape into the nucleus. It was also demonstrated that Tax can liberate p50/RelA NF-κB complexes held in the cytoplasm by the p100 precursor protein, in a manner analogous to that of IκB-α but independent of it. Tax physically interacts with p100, which may be the mechanism by which Tax antagonizes the cytoplasmic sequestration function encoded in the p100's ankyrin domain. A preferential *interaction of Tax with p100 has recently been described elsewhere; its func*tional significance, however, is reported to be related to viral latency because p100 retains Tax in the cytoplasm, which could preclude nuclear activities of Tax (Beraud et al 1994). The physiological importance of the Tax-mediated release of NF-κB complexes from p100-inhibited cytoplasmic pools remains to be shown. Release of NF-κB from such stores, together with induced proteolysis of IκB-α, may be required to maintain constitutively high levels of nuclear NF-κB.

HIV infection of monocytic cells has been reported to lead to activation of NF-κB after several days in culture (Bachelerie et al 1991; Riviere et al 1991; Paya et al 1992; Roulston et al 1992, 1993). This might ensure continued expression of integrated virus because such expression is dependent on NF-κB-induced transcription through *cis*-acting κB elements in the HIV LTR (Nabel & Baltimore 1987; Ross et al 1991). The mechanism of this activation is unclear. In apparent contradiction to virus-induced activation, the HIV Nef product has been reported as an inhibitor of NF-κB (Niederman et al 1992). Rather than activating NF-κB itself, the HIV-1 virus may depend on environmental cofactors to stimulate its propagation, because resting populations of primary cells fail to replicate HIV (Pantaleo et al 1993b). Sites of inflammation or immune reactivity may represent primary loci for viral spread (Embretson et al 1993; Pantaleo et al 1993a), possibly due to the presence of agents that activate NF-κB, such as the cytokine TNF-α, bacterial toxins, and neutrophil-generated H_2O_2 (see Table 2). Finally, co-infection of patients with the HTLV-I virus can act as a cofactor for HIV replication (Pierik & Murphy 1991), potentially through activation of NF-κB by Tax.

Other Activating Conditions

Okadaic acid and calyculin A, inhibitors of phosphatase 1 and 2A, cause activation of NF-κB (Thevenin et al 1990; Menon et al 1993). Both drugs lead to phosphorylation and proteolytic degradation of IκB-α (Y-C Lin & U Siebenlist, unpublished observations). It is possible that the action of phosphatase inhibitors reflects the direct and regulated involvement of a negatively acting phosphatase in the signal transduction to IκB-α. Alternatively, phosphatases may play an indirect role, for example, by continually counteracting basal, unstimulated activities of kinases that would otherwise activate NF-κB; in this model the phosphatase inhibitors would simply unmask basal activities. According to a recent report, phosphatase inhibitors, by themselves, only function in transformed cells, whereas primary cells require an additional signal such as H_2O_2 to activate NF-κB (Menon et al 1993).

In T cells, the phosphatase calcineurin has been shown to be an important component in signaling NF-κB. T cells typically require two apparently distinct signaling events for IL-2 expression and growth induction, a calcium signal and a signal mediated through PKC (Crabtree 1989). Whereas PMA, acting through PKC, can activate NF-κB weakly in Jurkat T cells, PMA and calcium ionophore act synergistically. Stimulation with PHA is critically dependent on the calcium signal, as first shown by the block to activation of NF-κB by the immunosuppressant cyclosporin A (CsA) (Schmidt et al 1990). This immunosuppressant and the structurally unrelated but functionally equivalent drug FK506 also inhibits the calcium ionophore-dependent synergy with PMA (Mattila et al 1990; Frantz et al 1994). Cyclosporin A and FK506, in combination with cyclophilins and FK-binding proteins (FKBP), respectively, are known to inhibit the calcium-calmodulin-dependent phosphatase calcineurin A (Schreiber & Crabtree 1992). Recently it was shown that a constitutively active calcineurin can replace the synergizing calcium signal for the inactivation of IκB-α (Frantz et al 1994). Calcium-dependent calcineurin apparently functions to augment various signals, and depending on the signal, this may be critical in T cells. The okadaic acid studies imply the existence of negatively acting phosphatase, while calcineurin plays a positive role in activation of NF-κB.

Mature B cells, unlike pre B cells, exhibit constitutive NF-κB activity (Sen & Baltimore 1986a,b). This suggests a possible developmental role for NF-κB factors including the rearrangement and the continuous expression of the immunoglobulin κ light chain locus through its κB element in the intronic enhancer (Lichtenstein et al 1994). Confirming these connections, v-abl, which transforms cells at the preB stage, has been reported to block NF-κB activity as well as κ light chain transcription and rearrangement (Klug et al 1994; Chen et al 1994). Stimulation and differentiation of 70Z/3 preB cells with LPS or IL-1 induces κ light chain gene expression through activation of NF-κB (Sen & Baltimore 1986b). Initially p50/RelA dimers are activated, but later these are replaced by p50/Rel dimers, which are maintained in the nucleus. Mature B cells typically contain activated p50/Rel dimers and sometimes activated RelB-containing dimers. p50/RelA dimers stay in the cytoplasm, where they remain sensitive to stimulation (Liou & Baltimore 1993; Miyamoto et al 1994).

How is NF-κB activity maintained in the nucleus of mature B cells? While the steady-state level of IκB-α is not reduced in these cells relative to unstimulated pre B cells, the turnover of IκB-α is significantly increased in mature B cells (Miyamoto et al 1994). IκB-α half-life is decreased and new synthesis of IκB-α is increased, presumably as a result of induced transcription of the gene encoding IκB-α by NF-κB itself (see Tax-mediated activation above). It is possible that a continuous signaling event in B cells leads to induced degradation of IκB-α and causes long-term activation of Rel complexes. The inhibition of Rel by IκB-α may be less efficient than that of RelA, at least under conditions of increased turnover of IκB-α. Continuous signaling in B cells may be due to weak autocrine or paracrine stimulation by cytokines like IL-1 and TFN-α, cytokines whose expression is positively controlled by NF-κB itself.

MUTUAL REGULATION OF NF-κB AND IκB-α

Induced Synthesis of IκB-α

Cellular stimulation leading to IκB-α proteolysis and nuclear translocation of NF-κB also results in the subsequent rapid induction of IκB-α mRNA and protein, commencing at about 20 min after initial signaling (Brown et al 1993; Scott et al 1993; Sun et al 1993; see also Rice & Ernst 1993; Chiao et al 1994; Read et al 1994). Transfection of cells with transactivating NF-κB subunits induces high levels of endogenous IκB-α mRNA (Brown et al 1993; Scott et al 1993; Sun et al 1993; see also Chiao et al 1994). The cloning and sequencing of the IκB-α promoter has confirmed the predicted presence of κB binding sites, which have been shown to mediate transcriptional stimulation by NF-κB (de Martin et al 1993; LeBail et al 1993; Chiao et al 1994). Therefore, transactivating NF-κB dimers potently induce their own inhibitor, IκB-α, which presumably is destined to restore the inhibited state (Brown et al 1993). This built-in feedback inhibition may assure a transient response once the initiating event fades, an essential feature for the regulation of genes whose functions may be harmful if expressed unchecked.

Removal of the TNF-α stimulus causes rapid inhibition of nuclear NF-κB activity by newly induced IκB-α, as evidenced by continued nuclear NF-κB if IκB-α synthesis is blocked, even in the absence of TNF-α signaling (Brown et al 1993; K Brown & U Siebenlist, unpublished observations). Such observations suggest that newly induced IκB-α can enter nuclei to inhibit the previously activated NF-κB as the stimulating signal fades. Although this has not been demonstrated directly, two arguments further support this contention. First, IκB-α when overexpressed in transfected cells and pp40 in v-rel-transformed lymphoid cells is readily observed in nuclei (Cressman & Taub 1993; Zabel et al 1993; Davis et al 1990). Uncomplexed IκB-α is presumably free to passively diffuse into the nucleus, a situation somewhat analogous to that which exists in untransfected cells during the peak of induced IκB-α synthesis (Brown et al 1993). Complexed IκB-α, on the other hand, is unable to enter the nucleus, possibly because NF-κB dimers and IκB-α mutually inhibit each other. Second, IκB-α can remove bound p50/RelA from its cognate recognition sites on DNA in vitro (Zabel & Baeuerle 1990).

Induced Synthesis of NF-κB Proteins

The precursor proteins p105 and p100 are induced after cellular stimulation with agents that also activate NF-κB (Bours et al 1990; 1992b). Nuclear run-on experiments have demonstrated a transcriptional upregulation in the case of p105 (Gunter et al 1989; Bours et al 1990). Although the induction of the precursors is delayed relative to that of IκB-α, the precursors' genes may nevertheless be directly induced by NF-κB as well (Sun et al 1994). The p105 gene promotor contains functional κB elements (Ten et al 1992; Cogswell et al 1993). Induced expression of the precursors presumably replenishes NF-κB stores after processing. Prior to processing, however, the induced precursors may be critical to limit activation since they would be expected to help retain any newly synthesized transactivating proteins like Rel or RelA in the cytoplasm, which may be an important action if IκB-α is continually degraded in response to stimuli.

In addition to p100 and p105, the expression of the gene encoding Rel is transiently upregulated in response to stimuli that activate NF-κB (Bull et al 1989), and the Rel promoter is known to contain a κB element (Hannink & Temin 1990; Capobianco & Gilmore 1991). RelB may be similarly regulated (see Ryseck et al 1992). The expression of RelA, on the other hand, is essentially constitutive, only small increases, if any, have been noted following various cellular stimulations (Molitor et al 1990), and there are no κB elements in the RelA promoter (Ueberla et al 1993). Induced expression of NF-κB subunits (with the exception of RelA) may be necessary to sustain nuclear NF-κB activity over longer periods of stimulation, as reported for HL60 cells (Hohmann et al 1991). Stimulation of Jurkat T cells over several hours results in increasing amounts of Rel relative to RelA in the nucleus, possibly because of preferentially induced levels of Rel (Molitor et al 1990; Doerre et al 1993). Cells expressing constitutively active NF-κB, such as B cells or HTLV I-transformed T cells, display primarily p50/Rel heterodimers in their nuclei (Liou & Baltimore 1993; Miyamoto et al 1994).

NF-κB-REGULATED GENES: The Promoter Context

Select Groups Of Genes Controlled by NF-κB

Table 3 lists many of the genes whose expression is controlled by NF-κB. In a few instances, this control remains to be demonstrated directly. NF-κB induces, at least in part, many effectors of immune, inflammatory, or acute phase responses. In particular, numerous genes encoding cytokines/growth factors/chemokines, biological messengers that control and coordinate the functions of many cells, are responsive to NF-κB. Some of these messengers can use NF-κB to amplify their signal by a positive autoregulatory loop; the pro-inflammatory cytokines TNF-α, TNF-β, and IL-1 not only activate NF-κB, but they themselves are induced by NF-κB (Messer et al 1990; Hiscott et al 1993; see also Baeuerle 1991). In the case of the TNF-α gene, the observed importance of NF-κB for its expression may depend on the cell type and the segment of the enhancer/promoter region analyzed (Goldfeld et al 1993; Ziegler-Heitbrock et al 1993). Other critical genes induced by NF-κB include the acute phase regulator IL-6, the antiviral IFN-β, and various chemokines that summon cells to sites of inflammation such as IL-8, Gro, and possibly also MCP-1, Mip1-α, and Rantes (Anisowicz et al 1991; Grove & Plumb 1993; Joshi-Barve et al 1993; Danoff et al 1994; Shattuck et al 1994; see also Baeuerle 1991; Grilli et al 1993).

Migration of immune cells into inflamed tissue involves adhesion to the blood vessel endothelium and subsequent extravasation. This process requires NF-κB-mediated induced expression of the cell surface adhesion proteins, endothelial leukocyte adhesion molecule 1 (E-selectin or ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (Iademarco et al 1992; Neish et al 1992; Kaszubska et al 1993; Shu et al 1993). The intercellular cell adhesion molecule 1 (ICAM-1) may also be under the control of NF-κB (Voraberger et al 1991). VCAM-1 and ICAM-1 are important in monocyte adherence to endothelial cells. Atherosclerotic lesions, which begin with monocyte depositions on blood vessel walls, may be caused by the NF-κB-induced inappropriate expression of cytokines and adhesion receptors on endothelial cells, possibly in response to oxidative stress signals, such as oxidized lipoproteins (Andalibi et al 1993; Collins 1993; Liao et al 1993; Parhami et al 1993). The association of NF-κB with adhesion is also suggested by experiments in which antisense RelA oligonucleotides (and in some instances antisense p50 oligonucleotides) administered to various cells, including embryonal stem cells, cause complete detachment from the substratum (Narayanan et al 1993; Sokoloski et al 1993). Also, PMA-induced adhesion of HL-60 cells could be inhibited by competitive binding of NF-κB in vivo (Eck et al 1993).

A number of viruses use the NF-κB transcription factor to regulate their own expression. Activation of NF-κB may be mediated by the virus or viral products, or it may depend on environmental signals (see above). CMV activates and is transcriptionally stimulated by NF-κB (Sambucetti et al 1989; Boldogh et al 1993; Kowalik et al 1993). Expression of HIV-1 is critically dependent on tandem κB sites in its LTR (Nabel & Baltimore 1987; Pierce et al 1988). This has been clearly demonstrated for the simian immunodeficiency virus (SIV) (Bellas et al 1993) in monocytes/macrophages but may also apply to HIV in these cells, primary T cells, and some T cell lines (Ross et al 1991). HIV-2 possesses only one κB element but contains sites for several other factors (Leiden et al 1992; Hannibal et al 1993).

In almost no cases does NF-κB act alone to regulate its target genes. Promoters or enhancers typically depend on multiple transcription factors for their activity. In some cases, the non-NF-κB factors may be constitutively active in cells, which makes NF-κB the sole mediator of signals. A recently emerging mechanism by which NF-κB and other DNA-binding factors may potently combine to induce transcription involves the direct physical association between these factors. Rather than simply combining the actions of their respective transactivation domains, cooperatively acting transcription factors can synergize to stimulate the process of transcription initiation. One consequence of physical interaction is cooperative DNA-binding on adjoining sites, essential for engagement of a weak binding site. Another consequence of physical interactions of bound factors may be to allow higher-order structures to form in which DNA is bent to bring upstream binding factors into close proximity with the basal transcription apparatus at the start site of transcription. Physical association of some factors with NF-κB may also result in negative regulation. A less obvious aspect of transcriptional control by association may be the selection of a particular transcription factor from among several that may otherwise compete for the same DNA site. Thus the particular NF-κB complex that binds a given κB site may be selected from among several by physical interaction with a transcription factor for an adjoining site.

Cooperative and Antagonistic Interactions With Multiple Transcription Factors

One of the best studied promoters/enhancers is that of the IFN-β gene, which is potently induced by viruses, in part through the activation of NF-κB (Hiscott et al 1989; Thanos & Maniatis 1992; Du et al 1993). Induction of IFN-β is an intracellular defense response against viral infection. Induction appears to be initiated by double-stranded RNA (dsRNA) (Visvanathan & Goodbourn 1989; Thanos & Maniatis 1992; Du et al 1993), probably through the action of the dsRNA-sensitive kinase (Hovanessian 1991). The IFN-β promoter contains a binding site for an ATF-2 homodimer and/or ATF-2/c-jun heterodimer, two sites for IRF-1 and a site for NF-κB (Miyamoto et al 1988; Harada et al 1989; Du & Maniatis 1992; Reis et al 1992; Thanos & Maniatis 1992; Du et al 1993); these sites lie next to each other in the order listed. In addition, the ATF-2 site is flanked by sequences recognized by the ubiquitous HMG I/Y, and the NF-κB site also harbors such a sequence within it (Thanos & Maniatis 1992; Du et al 1993). The current model envisions a cooperative interaction between HMG I/Y with NF-κB as well as with ATF-2. HMG I/Y facilitates binding by both transcription factors, most likely by protein-protein interactions, which have been demonstrated, and possibly also by this small protein's ability to bend DNA. Finally, ATF-2 and NF-κB interact with each other; this may bring the distal part of the enhancer bearing the ATF-2 complex next to the basal transcription factor apparatus, which lies close to the NF-κB complex, to facilitate transactivation by ATF-2 (Du et al 1993). The combination of factor interactions has one further critical consequence, namely the generation of a very stable protein-DNA complex at the promoter, perhaps a requirement for strong transactivation.

The potent induction of IFN-β by virus is most likely a consequence of the activation of all component parts, ATF-2, NF-κB, and IRF-1. Induction of IFN-β by other stimuli, for example by cytokines, may involve synergy between NF-κB and IRF-1 only (Fujita et al 1989; Abdollahi et al 1991). An analogous situation may exist in the MHC class I promoter, where adjoining binding sites for these two factors mediate the synergy of TNF-α (through NF-κB) and interferon-γ (through IRF-1) (Johnson & Pober 1994; Ten et al 1993) possibly by physical association (P Drew & K Ozato, unpublished observations).

The interplay of NF-κB with ATF factors appears to be critical for the induction of the E-selectin gene (ELAM-1) as well (Kaszubska et al 1993). This cell adhesion protein can be induced, for example, by cytokines, on endothelial cells where it acts to facilitate the binding and extravasation of neutrophils and a subset of leukocytes from the bloodstream into sites of inflammation (Collins 1993).

The interaction between NF-κB and ATF-2 may be analogous to the interaction between NF-κB and c-Jun and c-Fos, since all of these proteins that interact with NF-κB contain basic region plus leucine zipper (bZIP) domains (Stein et al 1993b) but are otherwise unrelated. In the case of c-Jun and c-Fos, it has been reported that their bZIP domain interacts with the RHD of p65 (Stein et al 1993a). Interaction between DNA binding/dimerization domains (such as bZIP and RHD) rather than between transactivation domains may explain cooperative DNA-binding. The functional synergy of NF-κB with c-Jun and/or c-Fos may be seen with either a κB binding site or an AP-1 binding site alone, i.e. only one of the factors is required to contact DNA, while the other appears to act as an accessory factor that does not bind DNA (Stein et al 1993a). It is not clear if such interactions occur in normal untransfected cells, or if these data reflect only what occurs when binding sites for these factors are appropriately juxtaposed on a given promoter element.

A functional interaction of NF-κB with basic helix-loop-helix transcription factors (bHLH) in the immunoglobulin κ enhancer may be expected due to the close proximity of their respective binding sites (Staudt & Lenardo 1991; Grilli et al 1993). This idea is supported by the demonstrated physical interaction of the *Drosophila* protein dorsal with several bHLH proteins (see below). Placement of the κ enhancer into *Drosophila* embryos results in striped patterns of expression determined by the interaction of dorsal and *Drosophila* bHLH proteins with the κB element and E boxes (mammalian bHLH-binding sites), respectively, on the mammalian enhancer (Gonzalez-Crespo & Levine 1994).

The Sp1 transcription factor acts in synergy with the NF-κB transcription factor to induce transcription of the HIV-1 LTR (Perkins et al 1993). Given that the spatial arrangement of the two respective binding sites is critical to this synergy, a direct interaction between these factors has been postulated. Sp1 is ubiquitous and is thought to act mostly in a constitutive manner.

The cytokine IL-6 and the chemokine IL-8 are synergistically induced by NF-κB and the C/EBP family of transcription factors through adjoining binding sites in their promoters (NF-IL6 is one member of the C/EBP family) (Kunsch & Rosen 1993; Matsusaka et al 1993; Stein & Baldwin 1993). C/EBP proteins, like CREB/ATF and Jun/Fos, are bZIP DNA-binding factors. The bZIP motifs of several C/EBP proteins have been demonstrated to interact with several RHDs (Le Clair et al 1992; Matsusaka et al 1993; Stein et al 1993b). Binding by C/EBP family members to cognate sites and the resulting transactivation are augmented in the presence of NF-κB, even in the absence of a κB element, while the reverse is true at κB sites; high exogenous expression of C/EBP proteins inhibits transactivation through κB sites (Stein & Baldwin 1993; Stein et al 1993b). In the context of the IL-8 promoter, NF-κB binding at the κB site allows C/EBP to cooperatively engage a relatively weak adjoining C/EBP binding site, which leads to synergistic transactivation (Stein & Baldwin 1993). The NF-κB complex acting on the IL-8 promoter may not be the classic p50/RelA complex but rather a RelA homodimer (and possibly a RelA/Rel heterodimer) (Kunsch & Rosen 1993; Stein & Baldwin 1993). A RelA/Rel complex has been implicated in the control of the urokinase gene (Hansen et al 1992, 1994). Synergistic activation by NF-κB and C/EBP proteins may also be important in the regulation of angiotensinogen (Ron et al 1990; 1991), serum amyloid A (Betts et al 1993), and nitric oxide synthetase (Lowenstein et al 1993; Xie et al 1994), all of which are expressed during acute phase reactions. The interaction of NF-κB and C/EBP transcription factors could be the basis for the more global synergy between the cytokines IL-1 and IL-6 in acute phase responses (Betts et al 1993), T cell activation, and immunoglobulin secretion because IL-1 activates NF-κB and IL-6 potently activates C/EBP.

The anti-inflammatory properties of steroids may be mediated in part by direct interaction with NF-κB. The induced expression of the cytokine IL-6, which is dependent on functional synergy between NF-κB and C/EBP-β (NF-IL6), is specifically downregulated by dexamethasone-activated glucocorticoid receptors (Ray & Prefontaine 1994). This inhibition is mediated through a direct physical association of RelA (p65) with the ligand-bound receptor. In support of such a complex, the dexamethasone-induced activation of the mouse mammary tumor virus is inhibited by overexpression of RelA (Ray & Prefontaine 1994). Thus the global antagonistic activities of steroids and inflammatory agents *could be explained by the physical complexing of steroid receptors* and NF-κB.

Dorsal and Dif

Dorsal is an essential morphogen that determines dorsoventral polarity in the developing *Drosophila* embryos (Govind & Steward 1991; Ip & Levine, 1992; St. Johnston & Nüsslein-Volhard 1992). Thus far no equivalent process or protein has been identified in development of vertebrates. However, NTera-2 embryonal carcinoma cells contain little or no NF-κB proteins but can be induced with the differentiating agent retinoic acid to express these proteins and their activities (Segars et al 1993). Also, early developing thymocytes contain activated NF-κB (Zuniga-Pflucker et al 1993). These observations raise the possibility that NF-κB may act in early mammalian development as well. Dorsal is activated to enter nuclei prior to cellularization in a gradient fashion, dependent on a ventrally generated positional cue. This protein is lacking in dorsal-most nuclei, and peak levels of nuclear dorsal exist on the ventral-most side. The diverse patterns generated by dorsal are infinitely more complex than the dorsal gradient might predict. Dorsal accomplishes distinct and sharp patterns of expression of various regulatory genes by a number of mechanisms including (*a*) cooperative interactions with other proteins, in particular bHLH proteins, (*b*) differential engagement of cognate binding sites, depending on the affinity of the site and the concentration of dorsal, and (*c*) direct repressive effects of dorsal in combination with unknown proteins or indirectly through a specifically induced repressor.

Ventral-most areas give rise to embryonal mesoderm and the dorsal-induced proteins Twist and Snail are essential to this process (Ip et al 1992b, Jiang et al 1992; Jiang & Levine 1993). Twist, a bHLH protein, requires high levels of dorsal for expression because of relatively weak dorsal binding sites in its promoter (Thisse et al 1988; Jiang et al 1992). This bHLH protein then cooperates with dorsal to assure strong and uniform expression of certain target genes, especially if they contain weak binding sites for dorsal and depend on both proteins, as does Snail. Snail is a zinc finger DNA-binding protein that functions as a repressor (Kosman et al 1991; Leptin 1991; Rao et al 1991; Ip et al 1992a,b; Jiang & Levine 1993). Since Snail depends on both dorsal and Twist for expression, it is expressed with a sharp border as a result of the multiple effects of a shallow dorsal gradient and a somewhat steeper Twist gradient (Kosman et al 1991; Leptin 1991; Ip et al 1992b). Rhomboid, a putative transmembrane receptor essential for differentiation of the ventral epidermis, is expressed only in ventral-lateral regions, the presumptive neuroectoderm (Bier et al 1990). Low levels of dorsal can induce Rhomboid expression through synergistic effects with juxtaposed binding sites for various bHLH proteins that include Daughterless, Achaete, and Scute (Ip et al 1992a; Gonzalez-Crespo & Levine 1993). Expression in the mesoderm is specifically repressed by Snail, which may physically overlap some of the binding sites for the bHLH proteins (Ip et al 1992a; Kasai et al 1992). Finally, dorsal directly represses ventral and ventrolateral expression of Zerknullt and Decapentaplegic, two proteins detected only in dorsal regions. This effect appears to be mediated by an unknown co-repressor that cooperatively acts with dorsal to repress gene expression across long distances on the chromosome (Jiang et al 1992; Kirov et al 1993). Whereas dorsal itself is intrinsically an activator, here it acts as a repressor. The mechanism for this is not known, but is likely to involve cooperative DNA binding interactions, as seen with bHLH proteins.

Recent discoveries in *Drosophila* have shown that the connection between regulation of defense systems and NF-κB is evolutionarily conserved. Insects display a rapid response to bacterial infection, including phagocytosis and elaboration of several diverse anti-bacterial proteins, like cecropins, attacins, diptericins, defensins, and lysozymes (reviewed by Boman et al 1991; Hultmark 1993). Many of the genes for these proteins are regulated by an inducible NF-κB-like factor through a κB-like element in their promoters. This was first discovered through studies of the cecropin and diptericin genes of the giant moth Cecropia (Sun et al 1991; Sun & Faye 1992; Engstrom et al 1993; Kappler et al 1993). This factor has now been cloned in *Drosophila* by homology to RHD proteins, and is termed Dif, for dorsal-related immunity factor (Ip et al 1993). The RHD of Dif is nearly as related to vertebrate Rel/NF-κB proteins as it is to dorsal. Dif is not expressed during early embryogenesis but rather during later stages of development. Nuclear Dif activity is inducible from cytoplasmic stores in cells of the fat body and in hemolymph. It is likely that cactus, the IκB inhibitor of dorsal, also functions as the inhibitor of Dif, given cactus's continued expression in adult tissues.

An ancient acute phase-like response is speculated to be the original function for the Rel/NF-κB/dorsal/Dif proteins (Ip et al 1993; Hultmark 1994).

Bcl-3: A Transcriptional Activator

Bcl-3 is a member of the IκB family of proteins, but recent evidence suggests that unlike IκB-α, this protein activates transcription through κB sites. Two mechanisms for activation have been proposed, an indirect one, in which Bcl-3 antagonizes inhibitory p50 homodimers (Franzoso et al 1992, 1993), and a direct mechanism in which Bcl-3 acts as an accessory factor, coupling with otherwise inert p52 homodimers to form competent transactivators (Bours et al 1993).

What is the evidence for an inhibitory role for p50 homodimers? Numerous transfection studies in a number of cells indicate that p50 homodimers are unable, by themselves, to significantly transactivate a variety of κB-dependent reporter constructs, and this is consistent with the lack of an identifiable transactivation domain on p50 (Schmid et al 1991; Schmitz & Baeuerle 1991; Ballard et al 1992; Bours et al 1992b, 1993; Franzoso et al 1992, 1993; Kunsch et al 1992; Mercurio et al 1992; Ryseck et al 1992; see also Fujita et al 1992, 1993; Kretzschmar et al 1992; Moore et al 1993 for opposing views). Indeed, p50 homodimers usually behave as transcriptional inhibitors in vivo, counteracting potent transactivation by p50/RelA heterodimers by direct competition for κB sites (Schmitz & Baeuerle 1991; Franzoso et al 1992, 1993). This inhibition is particularly noticeable for κB sites that display a preference for the p50 homodimers (Franzoso et al 1993). In resting, unstimulated cells, the ubiquitous p50 homodimers localize largely to nuclei where they may help to protect some genes against inadvertent induction by low levels of the potently transactivating NF-κB dimers (Franzoso et al 1993).

In vivo data further support an inhibitory role for p50 homodimers. The expression of IL-2 in resting, non-transformed mouse T cell clones is under negative control by p50 homodimers. Only a complete stimulation protocol with peptide and antigen presenting cells results in expression of IL-2 and correlates with the specific removal of p50 homodimers from DNA (Kang et al 1992). p50 homodimers also appear to inhibit expression of MHC class I genes. Highly metastatic cell variants, expressing few class I genes, display high levels of these homodimers, whereas the reverse is true for less tumorigenic variants (Plaksin et al 1993). It is conceivable, of course, that p50 homodimers, although apparently unable to transactivate by themselves, could do so indirectly by cooperating with other transcription factors in the context of a particular promoter.

Bcl-3 preferentially targets p50 homodimers and p52 homodimers. It not only interferes with the DNA binding of p50 homodimers, but also dissociates already bound homodimers from their cognate binding sites (Hatada et al 1992; Franzoso et al 1993; Nolan et al 1993). *Thus Bcl-3 can promote transcription by removing* tightly bound inhibitory p50 homodimers from DNA and freeing such sites for binding by potently transactivating heterodimers such as p50/RelA (Franzoso et al 1992, 1993). Such a situation may exist during mitogenic stimulation of primary T cells, where prebound p50 homodimers would be expected to block NF-κB heterodimers from engaging these κB sites, at least until the newly induced Bcl-3 *protein enters the nuclei to relieve inhibition (Franzoso et al 1993).* Preliminary evidence suggests that normal antigenic signaling of non-transformed T cells induces Bcl-3 (M Lenardo & U Siebenlist, 1994). Thus Bcl-3 could be the protein that removes p50 homodimers from the IL-2 promoter to allow expression of this cytokine. This form of regulation is very different from that of IκB-α in that it is positive and occurs in the nucleus.

Bcl-3 *can also participate directly in* transactivation through targeting of p52 homodimers (Bours et al 1993). p52 is structurally and functionally highly similar to p50, although its expression is generally lower, and homodimers are rarely observed, except in a few highly differentiated cells such as myelomas (Bours et al 1993; Chang et al 1994; G Franzoso & U Siebenlist, unpublished observations). Bcl-3 tightly associates with both homodimers, but unlike p50 *homodimers*, p52 homodimers are not efficiently dissociated from κB binding sites. Rather, Bcl-3 and p52 homodimers form ternary complexes with the κB sites. This enables these two proteins to induce transcription through the κB site, owing to transactivation domains residing on Bcl-3. Bcl-3 harbors two such domains located outside the ankyrin domain on the N- and C-terminal parts of the protein (Bours et al 1993). Significant transactivation by Bcl-3 and p50 homodimers is not observed, regardless of the p50 construct used for transfections, probably because of efficient Bcl-3-mediated dissociation of p50 homodimers from DNA (Bours et al 1993; G Franzoso & U Siebenlist, unpublished observations). However, others have suggested that Bcl-3 and p50 homodimers together can transactivate, albeit weakly (Fujita et al 1993). While direct transactivation by Bcl-3 through p52 homodimers is potent by comparison with p50 homodimers, it is conceivable that cell- or signal-dependent modifications of p50 or Bcl-3 (such as phosphorylation; see Nolan et al 1993) might also allow these molecules to transactivate.

It is interesting to speculate that the nuclear presence of p52 homodimers in some highly differentiated cells, together with constitutive expression of Bcl-3 (Bhatia et al 1991; Bours et al 1993; G Franzoso & U Siebenlist, unpublished observations), could result in transactivation of a limited set of genes in such cells, even in the absence of any stimulating signal. In this way, transactivation of select genes could occur by an IκB-α-independent pathway, a scenario that might be attractive to short-lived terminally differentiated cells.

NF-κB, TUMORIGENESIS AND APOPTOSIS

The v-Rel oncogene was isolated from the acutely transforming and replication-defective avian retrovirus REV-T. Infection of young chicks typically results in fatal tumors within 7 to 10 days. These tumors are primarily of hematopoietic origin, often with markers present for lymphoid or myeloid cells. Chicken embryo fibroblasts, splenocytes, and bone marrow cells can also be transformed *in vitro (Rice & Gilden 1988; Boehmelt et al 1992;* Bose 1992; *Morrison et al* 1992). In addition to various internal amino acid differences, v-Rel lacks the most C-terminal transactivation domain present on its cellular homologue Rel. Several conflicting theories have been advanced in the past regarding the mechanism by which v-Rel transforms cells and also regarding the potential of Rel to transform (Gilmore 1992; Boehmelt et al 1992; Bose 1992; Walker et al 1992). Recently, however, a new consensus has emerged: (*a*) Rel, *when overexpressed* in sufficient quantities, can transform some cells, albeit much more weakly than v-Rel (Hrdlickova et al 1994a; Kralova et al 1994). (*b*) C-terminal deletions of Rel, as well as particular RHD mutations in Rel, all enhance the tumorigenic potential. The C-terminal deletions eliminate a transactivation domain, as well as a cytoplasmic retention function; it is not known how this domain contributes to cytoplasmic localization. A similarly acting domain has also been noted *at the* C-terminus of dorsal; these domains may assist IκB-α-mediated inhibition (see above) (Hrdlickova et al 1994a; Kralova et al 1994; Nehyba et al 1994). (*c*) The amino acid changes in the most N-terminal part of the v-Rel RHD, as compared to Rel, may subtly affect the DNA binding properties relative to Rel, whereas mutations located within a region, including the C-terminal part of the RHD, may change the interaction with the IκB-α inhibitor (Diehl et al 1993). IκB-α was significantly less efficient in inhibiting DNA binding by Rel proteins that carry these internal mutations. This may help to establish an increased nuclear presence of v-Rel. (*d*) The transactivation domain that remains in v-Rel is necessary for transformation, which suggests that transcriptional stimulation of at least some target genes is required (Sarkar & Gilmore 1993). This refutes earlier scenarios in which the oncogenic properties of v-Rel were attributed to its apparent inhibition of p50/RelA-mediated transactivation, a phenomenon that could simply reflect a generally weaker transactivation potential of v-Rel, at least in most cells (see above) (Ballard et al 1990, 1992; Inoue et al 1991; Richardson & Gilmore 1991; McDonnell et al 1992). In support of a critical role for gene transactivation by v-Rel, this protein was reported to be a particularly potent activator of specific genes such as those coding for MHC class I and II proteins and IL-2 receptor-α (Boehmelt et al 1992; Hrdlickova et al 1994a, 1994b; Nehyba et al 1994).

The C-terminal transactivation domain of Rel, which is deleted in v-Rel, may normally play a role in the induction of anti-oncogenic functions such as apoptosis. Expression of high amounts of Rel was correlated with apoptosis and autophagocytosis in a large variety of cells in developing chick embryos. Furthermore, avian bone marrow cells expressing high amounts of Rel undergo a form of programmed cell death (Abbadie et al 1993). These effects appear to be cell specific because high expression in primary chick embryo fibroblasts transforms them and extends their life span (Abbadie et al 1993; Kralova et al 1994). The C-terminal deletion in v-Rel may prevent the induction of cell death in lymphoid cells because v-Rel transformed bursal lymphocytes are resistant to apoptotic stimuli like radiation, calcium ionophore, or dexamethasone (Neiman et al 1991).

Consistent with a transforming potential of Rel in avian cells, disruptions of the human Rel locus have been observed in several tumors, although no conforming pattern has been noted (Lu et al 1991). In addition to an association of v-Rel and Rel with avian tumors, the p52/p100 gene is translocated in a number of human B and T cell tumors and in particular in cutaneous lymphomas (Neri et al 1991; Fracchiolia et al 1993). In all cases, the translocations physically and functionally disrupt the ankyrin domain, which leads to proteins with an intact RHD and a remaining variably sized ankyrin repeat domain. These proteins are further processed into mature p52 proteins, and both forms are present and can be observed in nuclei, unlike the p100 precursor (Chang et al 1994; G Franzoso & U Siebenlist, unpublished observations) [although complexes of the precursor have been reported to have some DNA-binding activity (Potter et al 1993; Scheinman et al 1993)]. It is not known how the truncated p100 proteins contribute to tumorigenesis. It is possible that loss of a functional ankyrin domain significantly weakens cytoplasmic sequestration, thus allowing transactivating Rel/NF-κB dimers to more readily translocate to the nucleus.

As noted, Bcl-3 is another member of the NF-κB/IκB family that has been associated with rare forms of chronic lymphocytic leukemias (Ohno et al 1990). Bcl-3 is overexpressed in these tumor cells by its juxtaposition to the immunoglobulin enhancer. To date, no cell lines have been established from these leukemias, and it is not known how Bcl-3 contributes to tumorigenesis. However, based on the research cited above, one may predict a role for overexpressed Bcl-3 in inappropriate transactivation of specific κB-dependent target genes.

NF-κB AS A TARGET FOR THERAPIES

NF-κB in its various forms has many wide-ranging effects that are controlled by a complex regulatory network of inhibitors and co-activators. Given the intimate connection between defense reactions and NF-κB, this transcription factor and its regulators could provide central targets for therapeutic intervention in various diseases or pathological conditions such as toxic/septic shock, graft vs host reactions, acute inflammatory conditions, acute phase responses, radiation damage, atherosclerosis, and cancer. NF-κB could also be a therapeutic target against infections by viruses such as HIV-1. Specific targets for small synthetic inhibitors could be the transcription factor(s) itself or any of the essential signaling proteins including specific protein kinases and the protease(s) that digest IκB-α. In contrast to short-term treatments, a long-term systemic block of NF-κB activity, if desired, would likely severely suppress many important host functions. In these cases, a less broadly acting target for blocking may be advisable, such as that provided by the combination of NF-κB with certain other transcription factors in the context of a given promoter. In this way, limited sets of relevant genes could be inhibited.

ACKNOWLEDGMENTS

We thank all members of our laboratory for their many contributions to this review. We are grateful to Dr. Anthony S. Fauci for his continuing support. We apologize for being unable to cite many important contributions in this field due to the extremely large number of relevant publications. We thank M. Rust for her skilled assistance in the preparation of this review.

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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2642 - 2647, March 1997
Neurobiology

Transcription factor NF-kB is activated in primary neurons by amyloid b peptides and in neurons surrounding early plaques from patients with Alzheimer disease

(reactive oxygen intermediates y senile plaques y neuroprotection y H_2O_2 y neuronal cell death)

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Communicated by Charles Weissmann, University of Zurich, Zurich, Switzerland, December 30, 1996 (received for review February 19, 1996)

ABSTRACT Amyloid b peptide (Ab)-containing plaques are a hallmark of Alzheimer disease. Here, we show that the neurotoxic Ab, a major plaque component, is a potent activator of the transcription factor NF-kB in primary neurons. This activation required reactive oxygen intermediates as messengers because an antioxidant prevented Ab-induced NF-kB activation. Maximal activation of NF-kB was found with 0.1 mM Ab-(1 - 40) and 0.1 mM Ab-(25 - 35) fragments, making a role for NF-kB in neuroprotection feasible. Using an activity-specific mAb for the p65 NF-kB subunit, activation of NF-kB also was observed in neurons and astroglia of brain sections from Alzheimer disease patients. Activated NF-kB was restricted to cells in the close vicinity of early plaques. Our data suggest that the aberrant gene expression in diseased nervous tissue is at least in part due to Ab-induced activation of NF-kB, a potent immediate - early transcriptional regulator of numerous proinflammatory genes.

A characteristic of Alzheimer disease (AD) is the accumulation of a 40 - 43-amino acid peptide termed "amyloid b-peptide" (Ab or bA4) (1, 2). This peptide is the major component of senile plaques found in AD and in Down syndrome. Ab is a proteolytic fragment of the large amyloid precursor protein (APP) (3). Several animal models recently have underscored the importance of APP for neuronal function. A knockout mouse, in which a shorter exon 2-lacking form of APP is expressed, shows memory defects (4). Two APP-null mutation strategies recently have been developed (5, 6). APP-null mutation leads to impaired neuronal function and reactive gliosis (5). Overexpression of APP leads to the formation of senile plaques and synaptic loss (7). In addition, Ab was found to be neurotoxic for primary neurons and clonal cell lines (8 - 11). The neurotoxic action of Ab could be blocked by antioxidants (10) and relied on the production of reactive oxygen intermediates, e.g., hydrogen peroxide (11).

AD is accompanied by the expression of inflammatory cytokines and cell adhesion molecules, including interleukin 6 (IL-6), IL-1b, and intercellular adhesion molecule 1, in neurons and microglia (12). Several studies suggest that the neurodegenerative effects in AD can, at least in part, be attributed to the action of neurotoxic cytokines, such as IL-6 (13, 14). We have noticed that many of the genes newly induced in AD are under immediate - early transcriptional control of NF-kB, a factor present in neurons as well as microglia (15, 16).

Many mostly pathogenic stimuli can activate NF-kB (17), leading to the expression of a cellular defense program. In this line, activated NF-kB is a positive regulator of genes whose products mediate the acute phase response, lymphoproliferation, leukocyte adhesion, chemoattraction of macrophages, B and T cell activation, and antiviral response. Very recently, the neurotransmitter glutamate and its agonists kainate and *N*-methyl-D-aspartate were shown to cause activation of NF-kB in primary neurons (18, 19). Glutamate plays a role in both neurotransmission and neurodegeneration and has been shown to induce oxidative stress in neurons (20).

In nonstimulated cells, NF-kB is sequestered in the cytoplasm by inhibitory subunits called IkB proteins. Stimulation of cells by diverse inducers causes phosphorylation of IkB-a and subsequent degradation of IkB-a by the proteasome (21). Liberated NF-kB is transported in the nucleus, where it induces transcription of target genes, including IkB-a as an autoregulatory loop (21). Reactive oxygen intermediates (ROIs) can function as second messengers in NF-kB activation (22 - 25). Cytoplasmic NF-kB, containing p50 and p65 (RelA) DNA-binding subunits, was found in all regions of the brain (26) and in basically all cell types of the nervous system (16, 18, 27, 28). The p65 subunit is essential for survival (29).

Here we report that the neurotoxic peptide Ab is a potent inducer of NF-kB in primary neurons and astrocytes. This activity of Ab required the production of ROIs as messengers. Immunohistochemical analysis of brain sections from AD patients using a mAb with selectivity for the activated nuclear form of p65 revealed that NF-kB was activated in neurons and astrocytes. Cells with activated NF-kB were restricted to the close proximity of early plaque stages. We discuss the possibility that (*i*) Ab-induced NF-kB activation contributes to the pathological changes observed in AD via the induction of proinflammatory and cytotoxic genes or, more likely, that (*ii*) Ab-induced NF-kB activation is part of a cellular defense program.

MATERIALS AND METHODS

Primary Culture, Ab Treatment and Cell Survival Analysis. Cerebellar granule cell cultures were prepared from 6 - 7-day-old Wistar rats (18). At this stage, a-p65 mAb immunoreactivity in cerebellar neurons is very low (18). Ab-(25 - 35) (lots 506861 and 510178) and Ab-(1 - 40) (lots 506773 and 510598) (Bachem) fragments were dissolved in water at 1 mgyml

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Abbreviations: AD, Alzheimer disease; APP, amyloid precursor protein; Ab, amyloid b-peptide; IL, interleukin; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediate; TNF, tumor necrosis factor a; DAPI, 6-diamidino-2 phenylindole.

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directly before use or the solution was aliquoted and stored frozen at 2 208C. Refreezing was avoided. One batch of Ab-(1 - 40) tested for aggregation with Congo red staining and electron microscopy was obtained from Boehringer Ingelheim. A scrambled control peptide (sequence KSGNMLGIIAG; ref. 11) was synthesized and purified by HPLC. Substance P, bovine catalase, pyrrolidine dithiocarbamate (PDTC), and 3-amino-1,2,4-triazole were from Sigma. Inhibition of bovine catalase (specific activity 2200 units ymg protein) was done with a 5-fold molar excess of 3-amino-1,2,4-triazole for 1 h at ambient temperature followed by dialysis on Millipore filters. Cells were treated with the indicated concentrations of peptides in medium with serum for 45 min to 3 days. Thereafter, cells were fixed for 2 min in ethanol and for 5 min in 3.7% formaldehyde and immunostained. The sequence of the peptide used for antibody competition is described elsewhere (28). For analysis of neuronal survival in parallel to immunostaining, the fluorescent nuclear dye 6-diamidino-2-phenylindole (DAPI) was used. Nuclear chromatin morphology was analyzed with a 3 40 objective. Nonviable neurons were recognized by nuclear condensation and yor fragmented chromatin. In phase contrast images, those neurons were irregularly shaped with shrunken cell body and yor dystrophic neurites. The number of viable and nonviable neurons was counted in three to five fixed fields per chamber.

Immunostaining Methods. Isocortical tissue was obtained postmortem from patients with histopathologically confirmed AD. Cryostat sections (8 mm) were cut from frozen tissue using a Jung cryostat (Leica, Heidelberg) and mounted on gelatine-coated slides. Immunohistochemistry was performed essentially as previously described (15, 18, 28). For double labeling experiments, the brain sections were incubated with the two antibodies (diluted 1:50): an mAb against p65 (Boehringer Mannheim) and a polyclonal rabbit anti-Ab antiserum (anti-b amyloid, Alzheimer; Boehringer Mannheim). Bound antibodies were detected with a biotinylated anti-mouse IgG y avidin Cy3 complex and an anti-rabbit IgG coupled with dichlorotriazinyl amino fluorescein. In addition, 50 mm paraffin-embedded sections were cut from autopsy material obtained from the local Department of Neuropathology. Before immunohistochemistry, sections were treated for 10 min in a microwave oven in 0.1 M citrate buffer (pH 6.0). a-p65 mAb (1:50) was detected with a peroxidase-coupled secondary antibody using nickel-enhanced diaminobenzidine as substrate (Vectastain Elite, Vector Laboratories). Peptide preabsorbtion was done as described (18). Color slides for Figs. 1 - 4 were digitized using a Nikon Coolscan connected to an Apple Macintosh computer. Color slides for Fig. 5 were digitized with a Linotype-Hell Topaz Scanner at high resolution. Mounting of figures was done using ADOBE PHOTOSHOP software.

RESULTS

Nanomolar Concentrations of Ab Peptides Activate Transcription Factor NF- kB in Primary Neurons. We investigated by an immunocytochemical approach whether Ab could activate transcription factor NF- kB in primary cultures of cerebellar granule cells. A recently developed mAb (a-p65 mAb; ref. 28) was used that selectively stains the activated nuclear form of NF- kB and allows monitoring of NF- kB activation at the single cell level. The antibody recognizes an epitope on the DNA-binding p65 subunit, which is shielded by IkB in the inactive cytoplasmic form of NF- kB (28). Because the p65 gene is not rapidly inducible (30, 31), the appearance of nuclear p65 immunoreactivity results from the release of IkB during posttranslational activation.

An untreated control culture of cerebellar granule cells showed weak a-p65 mAb immunoreactivity, which did not overlap with the nuclei. Each image in Fig. 1, *a-c* shows a number of neurons (small nuclei) and an astrocyte (large nucleus). Treatment with 100 nM of Ab peptides [Ab-(1 - 40) and Ab-(25 - 35)] for 45 min induced a strong increase in p65 activity in both neurons and astrocytes (Fig. 1 *d* and *g*). a-p65 mAb immunoreactivity now colocalized with the nuclear DAPI staining (compare Fig. 1 *Top* and *Middle*). This shows that Ab can activate NF- kB in both neurons and astrocytes. Ab-(1 - 40) activated NF- kB in the same concentration range as Ab-(25 - 35), (see below) but substance P failed to activate NF- kB at concentrations up to 15 mM (data not shown). The concentration profile of the NF- kB activation induced by Ab-(1 - 40) (Fig. 2 *A*) and Ab-(25 - 35) (Fig. 2 *B*) was measured. Activation was quantified using histograms of full microscopic views, depicted on a linear scale with the relative amount of pixels shown on the ordinate and the fluorescence intensity shown on the abscissa (Fig. 2). We found that 100 nM reproducibly gave the strongest activation with both Ab-(1 - 40) and Ab-(25 - 35) resulting in a peak at the highest fluorescence intensity (Fig. 2 *A* and *B*, arrows). At a concentration of 1 mM Ab-(1 - 40), p65 was still activated (Fig. 2*A*) in contrast to neurons treated with 1 mM Ab-(25 - 35). Treatment with higher concentrations of peptides resulted in an induction of an a-p65 immunoreactivity, which no longer colocalized with the nuclear DNA staining but was concentrated in perinuclear aggregates. This was much more pronounced in cells treated with Ab-(25 - 35) (Fig. 2*B*).

Next, we tested whether the observed NF- kB activation by the Ab peptides was specific. A peptide of the same amino acid composition as Ab-(25 - 35) but with a randomized sequence (11) was tested at a concentration of 100 nM. The scrambled peptide failed to detectably activate NF- kB (data not shown). To assess the specificity of the a-p65 mAb, the antibody was preincubated with the p65 peptide used to raise the mAb (28). Peptide-preabsorbed a-p65 mAb did not stain granule cells treated with Ab (data not shown).

Activation of NF- kB by Ab Is Time-Dependent. Ab peptides were applied for several days on cerebellar granule cells. NF- kB activation was measured at different time points (data

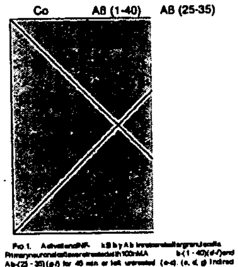

FIG. 1. Activation of NF- kB by Ab in cerebellar granule cells. Primary neuronal cultures were treated with 100 nM Ab-(1 - 40) (*d-f*) and Ab-(25 - 35) (*g-i*) for 45 min or left untreated (*a-c*). (*a, d, g*) Indirect immunofluorescence analysis of cell cultures for p65 NF-kB immunoreactivity using the activity-specific a-p65 mAb (28) and a Cy3-conjugated secondary antibody (see *Materials and Methods*). (*b, e, h*) DNA staining of nuclei made fluorescent using DAPI. (*c, f, i*) Phase-contrast micrographs. N, neurons; A, astrocytes. (Bar = 50 mm.)

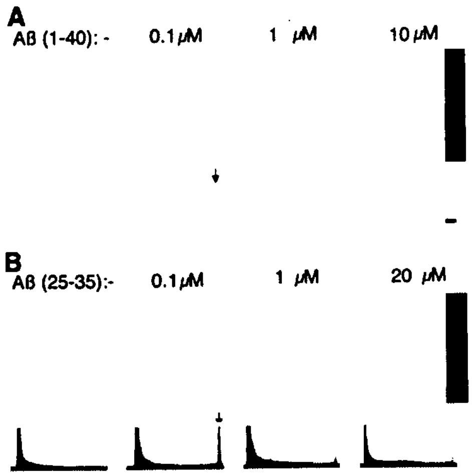

Fig. 2. Concentration dependence of Ab-induced NF-kB activation in cerebellar granule cells. (*A*) Granule cells treated with Ab-(1 - 40). (*B*) Granule cells treated with Ab-(25 - 35). (*Upper*) Indirect immunofluorescence analysis of primary cell cultures for binding of a-p65 mAb. Cells were stimulated for 45 min with the indicated concentrations of Ab-(1 - 40). Sections with two to four neurons are shown. Neurons were identified by DAPI staining and by their morphology upon phase contrast microscopy. Analysis of the immunofluorescence intensity of a larger area of the specimen in one optical plane. Histograms for each concentration are shown. The ordinate (linear scale) depicts relative amounts of pixels. The fluorescence intensities of the Cy3 chromophor are shown on a linear scale on the abscissa. Arrows indicate the maximal signals obtained at 100 nM Ab peptides.

not shown). NF-kB activation shows an inverted U-shaped concentration dependence for Ab peptides. Maximal activation was observed at a narrow concentration range of 0.1 mM Ab-(25 - 35) and 0.1 mM Ab-(1 - 40) up to 1 mM Ab-(1 - 40) after 1 h, and up to 24 h, of peptide exposure. No activation of NF-kB could be detected at higher concentrations even after an exposure of up to 3 days. Nevertheless, neurotoxicity of both Ab peptides (10 mM) was evident after 1 day of exposure and increased to more than 80% nonvital cells after 3 days. The neurotoxicity of the Ab peptides was even more pronounced when the treatment was done without serum.

Of interest, after 1 day of exposure with 0.1 mM Ab-(1 - 40) an up to 30% increase in viability of granule cells was observed. This correlates very well with the maximal activation of NF-kB at this neuroprotective dose of 0.1 mM Ab-(1 - 40) and is in a time frame to allow *de novo* gene expression. As observed with activation by other stimuli, e.g., tumor necrosis factor (TNF) or phorbol esters, NF-kB activation is a transient process, which is inhibited through *de novo* expression of the NF-kB-target gene I kB as part of an autoregulatory loop (21). To test the mechanisms involved in the repression of NF-kB activation at high concentrations of Ab, we tested several concentrations of H_2O_2 for NF-kB activation (data not shown). Surprisingly, here also an inverted U-shaped concentration curve was observed, and with H_2O_2 amounts higher than 40 mM, a perinuclear staining was evident. One explanation for this unusual localization of activated p65 might be an oxidative modification of p65 interfering with nuclear transport. Taken together, these data point to a role of NF-kB in neuroprotection rather than in neurodegeneration.

Activation of NF-kB by Ab Is Dependent on ROIs. Ab peptide solutions are capable of generating ROIs (11, 32), and ROIs were shown to be critical for the neurotoxic action of Ab, as was evident from the protective effect of antioxidants such as vitamin E or exogenously applied catalase (10, 11). Because NF-kB is activated by H_2O_2 in some T cell lines, in HeLa cells (23, 24), and in cerebellar granule cells, we tested whether ROIs, in particular H_2O_2, play a role in Ab-induced NF-kB activation. Cultures of granule cells were stimulated with Ab-(1 - 40) (Fig. 3, *a-d*, *Upper*) and Ab-(25 - 35) (Fig. 3, *a-d*, *Lower*) alone, in combination with the antioxidant PDTC (Fig. 3, *e-h*), an inhibitor of cellular H_2O_2 production in response to okadaic acid stimulation (33), and in combination with catalase (Fig. 3, *i-l*). PDTC (100 mM) almost completely blocked the appearance of Ab-activated p65 in nuclei (Fig. 3, *e* and *g*). Only a weak perinuclear p65 staining was evident.

TNF-induced NF-kB activation can be suppressed by stable overexpression of catalase or exogenously applied enzyme (25), suggesting that H_2O_2 is a critical ROI factor in TNF signaling. Because catalase also prevents the cytotoxic effects of Ab (11), we wondered whether NF-kB activation by Ab also was suppressed by the H_2O_2-degrading enzyme. Primary granule neurons were preincubated for 1 h with 400 mg/ml catalase before the addition of 100 nM Ab peptides [Fig. 3, *i-l*: Ab-(1 - 40) *Upper*; Ab-(25 - 35) *Lower*]. As shown in Fig. 3, *i-l*, exogenous catalase effectively suppressed the appearance of Ab-activated NF-kB in nuclei. In comparison to PDTC, inhibition with catalase is much less efficient. For inhibition of

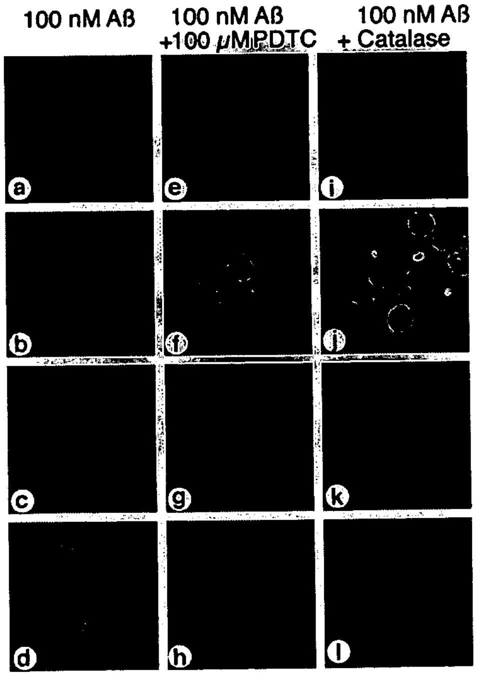

Fig. 3. The effect of the antioxidant PDTC and catalase on Ab-induced NF-kB activation. Granule cells were incubated for 45 min with 100 nM Ab alone [*a* and *b*, Ab-(1 - 40); *c* and *d*, Ab-(25 - 35)] or with Ab and 100 mM PDTC, which was added to cell cultures 10 min before Ab [*e* and *f*, Ab-(1 - 40); *g* and *h*, Ab-(25 - 35)], or with 100 nM Ab plus 400 mg/ml catalase, which was added to cells 1 h before the Ab peptide [*i* and *j*, Ab-(1 - 40); *k* and *l*, Ab-(25 - 35)]. Cell cultures were analyzed by indirect immunofluorescence for an increase of a-p65 mAb immunoreactivity (*a*, *e*, *i*, *c*, *g*, *k*) and by nuclear DAPI-staining (*b*, *f*, *j*, *d*, *h*, *l*).

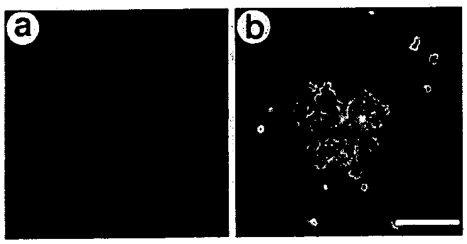

FIG. 4. Colocalization of a-p65 mAb and anti-A b immunoreactivities in senile plaques from brains of AD patients. In double immunofluorescence experiments, cryosections from patients with AD were immunostained with polyclonal anti-A b (*b*) and monoclonal anti-p65 (*a*) antibodies. Bound antibodies were detected by species-specific secondary antibodies conjugated to either Cy3 [red fluorescence, anti-p65 (*a*)] or dichlorotriazinyl amino fluorescein [green fluorescence, anti-A b (*b*)]. (Bar 5 10 mm.)

TNF-mediated NF-kB activation, a prolonged pretreatment (30 h) with exogenous catalase was necessary (25). Here, only a 1-h pretreatment with catalase was needed to clearly suppress NF-kB activity. We tested also catalytically inactive catalase. Therefore we preincubated catalase with the specific inhibitor 3-amino-1,2,4-triazole (25). The treated catalase was tested for inhibition of A b-(1 - 40)- and A b-(25 - 35)-mediated NF-kB activations. Surprisingly, the inactivated catalase acted also as an inhibitor of NF-kB activation (data not shown). One possible explanation might be an interference of catalase with A b aggregation.

NF-kB Immunoreactivity Is Present in Senile Plaques. We investigated whether NF-kB is activated in the vicinity of A b-containing plaques in the brains of AD patients. The first series of experiments were performed with cryosections from brains of patients with AD stained by an indirect immunofluorescence technique because of this technique' s suitability for double labelings. A total of 10 sections originating from the limbic cortex and hippocampus of four AD brains were analyzed. Bindings of polyclonal anti-A b antibodies and of a monoclonal a-p65 antibody were visualized by respective, specific secondary antibodies. In Fig. 4*a*, red fluorescence (Cy3) corresponds to activated p65 and green fluorescence (dichlorotriazinyl amino fluorescein) corresponds to anti-A b immunoreactivity (Fig. 4 *b*). An example of a senile plaque, as detected by an anti-A b antiserum, is shown in Fig. 4 *b*, and the same structure is stained with a-p65 in Fig. 4*a*. Out of 300 anti-A b immunoreactive plaques inspected, ' 65% were found to also be strongly positive for a-p65 immunoreactivity. No staining was evident when the mAb was preabsorbed with the corresponding p65 peptide epitope (data not shown).

Early Plaque Stages Are Surrounded by Neurons with Activated NF-kB. Using paraffin-embedded sections of AD brains and peroxidase-conjugated anti-mouse-IgG, distinct plaque stages in the isocortex of AD patients were analyzed for a-p65 mAb immunoreactivity. Although this procedure is of lower sensitivity compared with immunofluorescence staining methods, as evident from a lower density of NF-kB-positive plaques, it has the advantages that lipofuscin granules are not detected and that different plaque stages can be identified. We have analyzed diffuse, primitive, and classical plaques in a total of 30 samples from the cerebral cortex of four patients with AD. A total of 100 plaques of each sample was inspected. The majority of plaques was significantly labeled with a-p65 mAb (see Fig. 4) whereby the intensity of labeling correlated with the plaque stage. The strongest staining was observed in primitive plaques, and diffuse and classical plaques showed only weak p65 staining (data not shown). In primitive plaque stages, a-p65 mAb immunostaining clearly labeled plaques (Fig. 5, brown color). Two examples of regions with primitive plaques are shown at low power view in Fig. 5 and are consistent with the results from indirect immunofluorescence labeling (see Fig. 4). A number of cells surrounding the plaque (Fig. 5, arrows), among them pyramidal neurons (identified by their triangular shape at high power magnification; data not shown), showed a strong nuclear a-p65 mAb immunoreactivity (dark brown) indicating both the presence of activated transcription factor NF-kB and an inducing signal derived from the nearby primitive plaque. In brain regions free of senile plaques, very few anti-p65 immunoreactive cells were observed (Fig. 5, asterisks). This observation in postmortem tissue was in contrast to freshly prepared rodent brains, which have a relatively high density of a-p65 mAb-positive neurons (15, 28) and may reflect ceased neuronal activity. Taken together, our data suggest that predominantly early plaque stages can emit

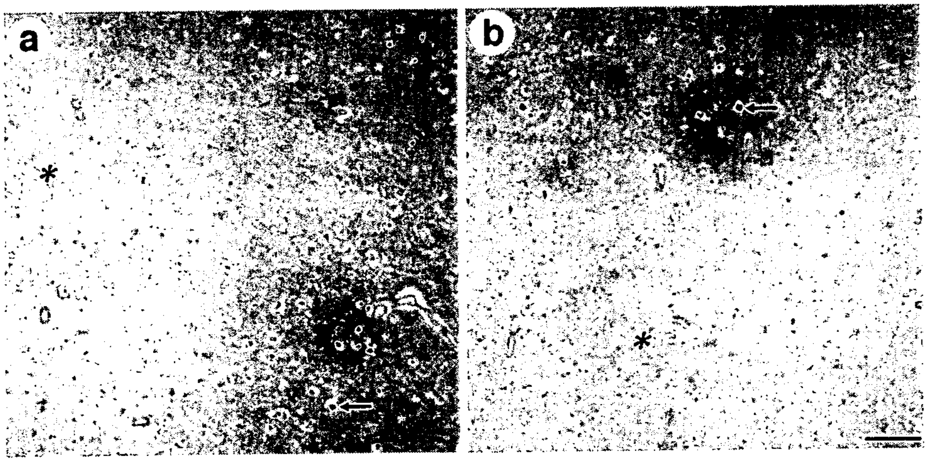

FIG. 5. Immunohistochemical analysis of plaques in cortical sections of AD brains for a-p65 mAb immunoreactivity. Plaques are visualized by immunoreactivity (brown color). Examples of a-p65 immunoreactive cell nuclei (arrows) and surrounding plaques are depicted. Different examples for primitive plaque types are shown. Regions distant from plaques have reduced a-p65 immunoreactivity (asterisks). (Bar 5 50 mm.)

a signal activating NF-kB in closely adjacent neurons and astroglia of the cerebral cortex of patients with AD.

DISCUSSION

The Mechanism of NF-kB Activation by Ab. In this study, we show, in primary neuronal cultures, that the transcription factor NF-kB can be potently activated by nanomolar concentrations of both neurotoxic peptides Ab-(1-40) and Ab-(25-35). Cerebellar granule cells were used as a convenient well characterized model of cultured neurons. Similar activation of NF-kB was detected in postmortem sections from cortex and hippocampus of patients with AD, suggesting that this response was not limited to cerebellar neurons. The Ab-(25-35) peptide has been shown to induce oxidative stress in primary neurons and to activate a kB-dependent reporter gene construct in transiently transfected B12 neuroblastoma cells (11). Here, we directly observed NF-kB activation at a single cell level in both cultured primary neurons and astrocytes and provide evidence that this also may occur in a pathological *in vivo* situation.

It has been reported that the neurotoxic action of Ab relies on H_2O_2 production (11). Here we show that NF-kB activation by Ab was prevented by PDTC, a compound shown to prevent cellular H_2O_2 production and the *de novo* phosphorylation and subsequent degradation of IkB-a in response to TNF and IL-1 (34, 35). We also found that the Ab-mediated NF-kB activation could be blocked by exogenous catalase. This might be a result of a catalase catalyzed degradation of H_2O_2. But, surprisingly, catalase treated with the specific inhibitor 3-amino-1,2,4-triazole also blocked NF-kB activation. A nonenzymatic mechanism for catalase in inhibiting Ab-mediated NF-kB activation also is supported by the short time of preincubation (1 h), which is sufficient for blocking, whereas TNF-mediated NF-kB activation is only blocked after a prolonged pretreatment (30 h) with exogenous catalase (25). Taken together, these data suggest a structural interaction of catalase with Ab peptides, independent of enzymatic activity. The consequence might be an interference with Ab aggregation and yor receptor binding.

Ab has been reported to generate ROIs in the absence of cells (32), so we tested exogenous H_2O_2 for NF-kB activation. In HeLa and Jurkat T cell clones, 50-250 mM of exogenously applied H_2O_2 was required to activate NF-kB (23, 24). Corresponding to the inverted U-shaped activation curve of Ab, 10-40 mM of H_2O_2 was efficient in activating NF-kB in cerebellar granule cells. Perinuclear aggregates of p65 could be detected in cells exposed to high amounts of Ab or H_2O_2. One possible explanation for this unphysiological location is an accumulation of covalently modified NF-kB due to increased oxidative stress. Future studies with purified proteins will further address this question.

Recently, it was shown that Ab interacts with tachykinin receptors (36). However, substance P, one of the natural ligands of tachykinin receptors, failed to activate NF-kB in primary cerebellar granule cells, suggesting that tachykinin receptors were not involved in NF-kB activation by Ab.

Activated NF-kB in Brains of Patients with AD. In this study, we provide immunohistochemical evidence that Ab may activate NF-kB in brains from patients with AD. It must be emphasized that postmortem material was analyzed, with an autolysis time up to 12 h. During this time, the IkB-shielded epitope for a-p65 mAb could become exposed on p65 because of enhanced proteolysis. However, a major argument against such an artificial NF-kB activation is that very few cells in cryosections and paraffin-embedded sections were immunoreactive, except for those in the close vicinity of certain plaque stages (see Fig. 5).

Two types of a-p65 mAb immunoreactivity were observed in AD brains. First, early plaque stages were directly stained, in particular primitive plaques, which showed a strong reactivity in their center. The same staining pattern was reported for the inflammatory cytokine IL-6 (14), whose gene is known to be inducibly controlled by NF-kB (12). Likewise, IL-1 and intercellular adhesion molecule 1, which are both encoded by target genes for NF-kB (12), were found in plaques (37, 38). It has been suggested that the center of classical plaques contains an activated microglial cell, producing inflammatory cytokines such as IL-6 and IL-1 (39) that could be produced as a consequence of activated NF-kB. Recently, the APP gene was identified as a NF-kB target gene (40), explaining the upregulation of this gene by IL-1. In addition, Ab-(25-35) can stimulate the expression of IL-1 but not of IL-6 (41). The whole system consists of several feedback loops, like IL-1 and APP, which stimulate their own synthesis directly or indirectly via microglia activation (39). In this system, NF-kB seems to function as a central regulator. NF-kB is present in synapses (19, 26), so the p65 immunoreactivity found within plaques may alternatively (or in addition) originate from disintegrated synapses (42). The absence of nuclear DAPI staining from the plaque center (data not shown) would support this model.

Second, a-p65 mAb immunostaining was seen in neurons and astroglia surrounding primitive plaques. These discrete nuclear stainings indicate the exposure of cells to a strong NF-kB-activating signal originating from the plaque. Ab is the major plaque component, so Ab is a likely candidate. The reduced p65 immunostaining in the vicinity of classical plaques could come from the progressive loss of neurons by apoptotic cell death (43).

In the context of a neurodegenerative disease, our study makes a link among one causative agent (Ab), production of secondary ROI signals, activation of NF-kB, an ROI-inducible transcription factor, and expression of a number of inflammatory genes known to be regulated by NF-kB. Below we discuss the possibility that the activation of NF-kB could initiate a neurodegenerative or neuroprotective gene expression program, depending on the cellular context.

A Possible Role for NF-kB in Neurodegeneration. Do such toxic compounds as ROIs really depend on a transcription factor to exert their neurotoxic effects? High ROI doses may not need transcriptional events; however, a chronic exposure to low ROI doses may depend on a genetic program to exert a neurotoxic effect. Upon oxidative stress, NF-kB may induce the expression of gene products with a direct or indirect neurotoxic activity. Such indirect neurotoxic effects might well be the consequence of NF-kB activation in glial cells. On the other hand, candidates for neuronal NF-kB target genes with a direct neurodegenerative effect are the inducible NO synthase (44) and IL-6 (45). Novel major histocompatibility complex class I expression in stressed neurons could have an indirect neurodegenerative effect by targeting the cells for immunosurveillance (46). Of interest, stimulation of *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate receptors on cerebellar granule neurons leads to a rapid activation of NF-kB and a subsequent surface expression of major histocompatibility complex class I molecules (18).

A Possible Role for NF-kB in Neuroprotection. Depending on the genetic program of a cell, NF-kB may be able to activate expression of genes with neuroprotective function, e.g., in neurons, whereas neurodegenerative functions are directed in glia. This could explain why both TNF a and b can induce neuroprotection against Ab-induced neuronal death when applied 24 h before Ab (47). Recently, we found that nerve growth factor can activate NF-kB via the p75 neurotrophin receptor (48). Of interest, this receptor is abundantly expressed on cholinergic neurons and is necessary for sprouting after lesions (49).

A further possibility is that short term stimuli of NF-kB lead to neuroprotection whereas chronic stimuli, e.g., long term incubation with Ab peptides, cause NF-kB inhibition and subsequent neurodegeneration. A link of NF-kB activation and neuroprotection is supported by the following observations: (*i*) The strongest NF-kB activation is only found at extremely low concentrations of Aβ (maximal at 0.1 mM); (*ii*) this inverted U-shaped activation curve is found with both Aβ-(25-35) and Aβ-(1-40); (*iii*) for Aβ-(1-40), a concentration of 0.1 mM is neuroprotective in cerebellar granule cell paradigm used here; and (*iv*) *in vivo* only, early plaque types are surrounded by neurons with activated NF-kB.

Taken together, these data underscore a role for NF-kB as an immediate sensor for increased levels of ROIs in neurons. In response to increased ROI levels, NF-kB could direct the expression of a cellular defense program, as shown for the immune system. Under chronic stimulation and yor stimulation with high amounts of Aβ, this sensing system seems to be overloaded, with the consequence that there will be no increase in cellular buffering mechanisms for ROIs, and this might result in further exacerbation of the neurodegenerative process.

Transcription factor NF-kB is activated by Aβ, ROIs, and inflammatory cytokines in various cell types of the brain and induces a number of genes with relevance for AD. Hence, the factor may play an important role in the development of the disease and define a novel drug target for slowing down or arresting the progression of AD.

We thank A. Schulze-Specking for superb technical assistance. Dr. Hubertus Stockinger (Boehringer Mannheim) is gratefully acknowledged for providing α-p65 mAb. Dr. Franz-Josef Schneider (Boehringer Ingelheim) is thanked for providing Aβ-(1-40). This work was supported by grants from the Bundesministerium für Bildung, Wissenschaft, Forschung, und Technologie (Schwerpunkt Autoimmunität), Volkswagen-Stiftung, and Sonderforschungsbereich 505 of the Deutsche Forschungsgemeinschaft.

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The NF-κB transcription factor induces DNA bending which is modulated by its 65-kD subunit

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Received September 11, 1990; Revised and Accepted October 16, 1990

ABSTRACT

This study describes a novel functional property of the eukaryotic transcription factor NF-κB in altering the DNA structure. We observed that the binding of purified NF-κB to DNA was facilitated by spermine and cations which are known to promote DNA bending, and by a nick in one position at the 3'-end of the binding site. Furthermore, the position of the NF-κB binding sequence 5'-GGGACTTTCC-3' (κB motif) within circularly permutated DNA fragments had a profound influence on the mobility of NF-κB-DNA complexes in a gel retardation assay while the mobility of unbound DNA fragments did not depend on the position of the κB motif. The mobility effect was slightly reduced at increased temperature. These observations suggested that binding of NF-κB to DNA induces bending. The estimated bending angle induced by the 50-kD DNA binding subunit of NF-κB (p50) was smaller than that induced by p50 which was associated with the 65-kD non-DNA binding subunit (p65). Moreover, the presence of p65 appeared to result in a shift of the bending center from the middle towards the 3'-end of the κB motif. This shows a role for the p65 subunit of NF-κB in modulating the extent and altering the position of protein-induced DNA bending.

INTRODUCTION

The NF-κB transcription factor can induce the expression of genes in different cell types by binding to decameric sequence motifs in promoter and enhancer elements (for reviews see 1–3). Most of its target genes encode proteins that are rapidly induced as part of immune and acute phase responses and during inflammatory processes. These proteins include immunoreceptors, acute phase proteins, cytokines and cytokine receptors. NF-κB can very rapidly activate genes because the protein is already present in unstimulated cells and requires for its activation only the release from its inhibitory subunit IκB and translocation from the cytoplasm into the nucleus (4). Viruses, T cell mitogens, bacterial lipopolysaccharide, cytokines and various other agents can activate the transcription factor (for reviews see 2,3). Phosphorylation of IκB by protein kinases appears to be an important trigger for the activation of NF-κB (5,6).

NF-κB exists as various multiprotein complexes. The simplest DNA binding form of NF-κB is composed of a dimerized 50 kd protein (p50) (7). It can be obtained from a heterotetrameric complex of NF-κB, which contains in addition to p50 two 65 kd non-binding subunits (p65), by electrophoretic separation of p50 under denaturing conditions followed by its renaturation. At present, it is unclear whether the p50 dimer has a physiological relevance or is an artifact. The prominent DNA-binding form of NF-κB in nuclei of activated cells is the heterotetramer. The non-DNA binding cytoplasmic NF-κB from unstimulated cells is a heterotrimeric complex composed of p50, p65 and IκB (8). Presumably, IκB prevents in this complex that two p50-p65 heterodimers can assemble to a heterotetramer. The p65 subunit serves as a receptor for the inhibitory subunit IκB (9) and is therefore required by NF-κB to become inactivated again by IκB. This can occur even when NF-κB is bound with high affinity to DNA (8).

An additional role of p65 in the nuclear form of NF-κB is to modulate the DNA-binding specificity of p50 (9). While, in the absence of p65, the p50 dimer can bind with high affinity to completely palindromic binding sequences, the heterotetramer binds with a 10- to 20-fold lower affinity to these sites but shows a two-fold increased affinity for the less symmetric κB motif 5'-GGGACTTTCC-3'. It is not yet understood how the multiprotein complex of NF-κB can initiate the synthesis of mRNA as a consequence of its binding to enhancer and promoter elements, and whether p65 is required for transcriptional activation.

In this study we have investigated whether binding of NF-κB alters the structure of DNA. A distortion of DNA structure that in prokaryotic systems is apparently involved in the control of transcription is DNA bending (10,11). Bending occurs either as an intrinsic property of DNA or is induced after sequence-specific binding of proteins (for reviews see 12–14). Here we report that DNA is strongly bent upon binding of NF-κB and that the non-DNA binding 65 kD subunit of NF-κB modulates the extent and alters the position of DNA bending.

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RESULTS

Polyamines and metallic cations facilitate binding of NF-κB to DNA

Using electrophoretic mobility shift assays (EMSAs), we have tested the influence of polyamines and various metallic cations on binding of NF-κB to an oligonucleotide encompassing the κB binding motif 5′-GGGACTTTCC-3′ (Fig. 1A). The NF-κB was DNA affinity-purified from cytosol of human placenta after its activation by a low pH-treatment (8). It was composed of both 50 and 65 kD protein subunits (referred to as heterotetrameric NF-κB) and was indistinguishable from active nuclear NF-κB (15). In the EMSAs shown in Figure 1 (A and B, lane 1) a concentration of NF-κB was used which gave only a barely detectable amount of protein-DNA complex. A dramatic increase in the amount of the complex was obtained if spermine was included in the binding reactions (Fig. 1A, lanes 2–5). Optimal complex formation was seen with 0.5 mM spermine. Spermidine had a similar although weaker effect (data not shown). Spermine has been reported to promote and stabilize structural alterations of DNA such as bending (16). It is therefore possible that the increased binding of NF-κB to DNA in the presence of spermine resulted from a structural alteration of the target DNA sequence by the polyamine. Alternatively or in addition, polyamines could exert their effect by stabilizing the protein subunit structure of NF-κB.

We also tested various metal cations which have been reported to promote DNA bending (16,17) for their effect on the DNA binding of NF-κB (Fig. 1B). While 1 to 10 mM Mg^{2+} barely increased DNA-binding of the transcription factor (Fig. 1B, lanes 2 to 4), Ca^{2+}, Ba^{2+} and $Co^{3+}(NH_3)_6$ cations induced a significant dose-dependent increase in the amount of the NF-κB-DNA complex (lanes 5 to 13). The maximal effects were seen with 5 mM Ca^{2+}, 1 mM Ba^{2+} and 25 μM $Co^{3+}(NH_3)_6$. These concentrations were similar to those at which the metal cations were previously shown to exhibit optimal alteration of DNA structure. The enhancing effect by both polyamines and various metallic cations suggests that the interaction of NF-κB with DNA is facilitated by modification of the cognate DNA structure rather than by a direct interaction of the agents with the protein.

Selective binding of NF-κB to nicked DNA

Support for the idea that bending or kinking of DNA facilitates binding of NF-κB came from a hydroxyl radical missing contact analysis (19). This method allows to investigate the effect of specific nicks in the DNA backbone on the binding of proteins. For that purpose, the DNA was OH-radical treated prior to its use in EMSAs. It is not yet clear whether the OH-radical treatment solely nicks DNA or, in addition, removes a nucleoside. The ^{32}P-end-labeled DNA fragment used in an EMSA (Fig. 2A, lane 1) showed a regular accessibility for hydroxyl radicals (Fig. 2A, lane 5; 2B, panel 1) and no strikingly unusual DNase I digestion pattern in the κB motif suggesting that the NF-κB binding site had no unusual structure prior to the binding of protein (19,20)(Fig. 2A, lane 2). The conditions for the OH-radical treatment were chosen such that on an average only a single nick per DNA molecule was produced. Among the OH-radical treated DNA-fragments that were bound by NF-κB, the amounts of those species were found decreased which were damaged by a nick within the decameric binding motif 5′-GGGACTTTCC-3′ (Fig. 2A, lane 6; 2B, panel 2). There was no single position within this binding motif where a nick could completely prevent the interaction of NF-κB with DNA (Fig. 2B, panel 2). Missing contacts in the 3′-half site of the decameric motif interfered with binding of NF-κB more strongly than those in the 5′-half site, as is evident from the scan shown in Fig. 2B (panel 2).

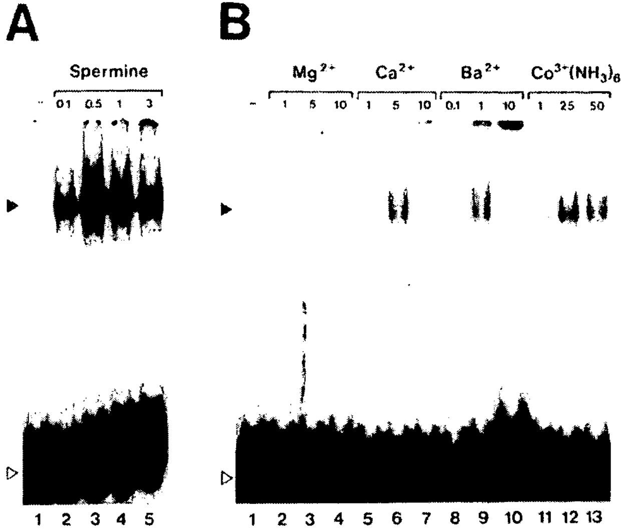

Fig.1. The effects of spermine and metallic cations on the DNA binding of purified NF-κB. (A) The indicated concentrations of spermine in mM were included in a standard DNA binding reaction (15). followed by addition of highly diluted affinity-purified heterotetrameric NF-κB (less than 20 pg of protein). The control (lane 1) was run on the same gel. The ^{32}P-endlabeled DNA probe (0.1 ng) was a synthetic double-stranded 30-mer oligonucleotide encompassing sequences from the κ light chain enhancer (20). (B) The indicated concentrations of the chloride salts of Mg^{2+}, Ca^{2+} and Ba^{2+} (all in mM) and of $Co^{3+}(NH_3)_6$ (in μM) were used in EMSAs. Fluorograms of native gels are shown. The filled arrowhead indicates the position of the NF-κB-DNA complex and the open one the position of unbound DNA probe.

A DNA fragment that was nicked in the next position downstream of the 3′-end of the binding motif was found highly enriched in the NF-κB-DNA complex and was present in a reduced amount in the fraction containing unbound DNA (Fig. 2A, compare lanes 6 and 7; 2B, compare panels 2 and 3). It appears that the binding of NF-κB to its cognate DNA was greatly facilitated if there was a nick at the 3′-end of the motif. The result with NF-κB is reminiscent to one obtained with the bacteriophage 434 repressor which recognizes an operator sequence with a single nick in its center with a higher affinity than the intact site (21).

NF-κB induces an asymmetric bending of the κB motif

The above observations prompted us to test directly whether NF-κB is capable of inducing a DNA bend or kink, or of increasing locally the flexibility of DNA. Circular permutation mobility shift assays (CP-EMSAs) were used in many instances to demonstrate that certain DNA sequences, or the binding of proteins to DNA, cause an alteration of DNA structure (22,23). An oligonucleotide encompassing the NF-κB binding site 5′-GGGACTTTCC-3′ and flanking sequences from the mouse κ light chain enhancer was inserted into a vector allowing circular permutation of the site (24). Eight 146 bp long restriction fragments (A-H) were isolated from the vector pUC-κB-Bend which contained a single NF-κB binding motif in various positions (Fig. 3A). After ^{32}P-endlabeling, the DNA fragments were used in EMSAs to form complexes with the heterotetrameric form of NF-κB. A strong

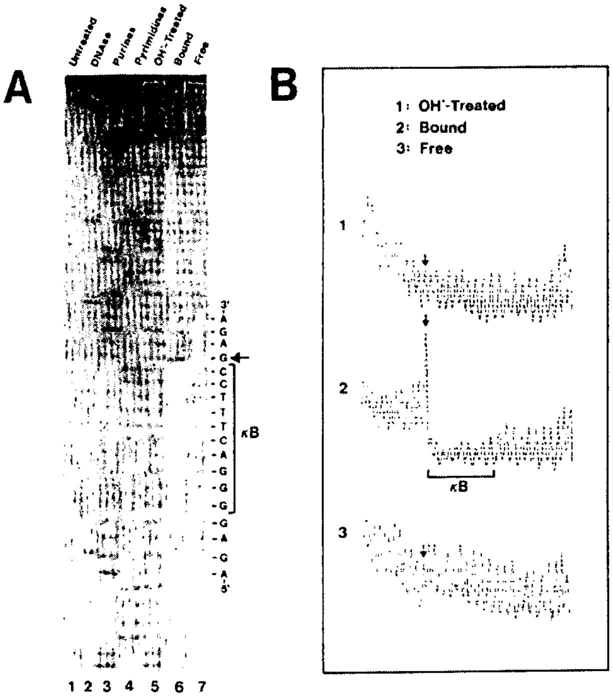

Fig.2. Missing contact analysis of NF-κB DNA binding. (A) Purified NF-κB (approximately 7 fmoles) was incubated with a hydroxyl radical-treated DNA-fragment (lane 5; 70 fmoles). The DNA binding reaction was subjected to native gel electrophoresis. Bound (lane 6) and free DNA (lane 7) was isolated and processed as described (37). DNA was treated as indicated on top of the lanes and subjected to electrophoresis on denaturing gels. Fluorograms are shown. (B) Scans of fluorogram lanes 5, 6 and 7. The arrows indicate the position of a G residue the absence of which facilitated NF-κB binding.

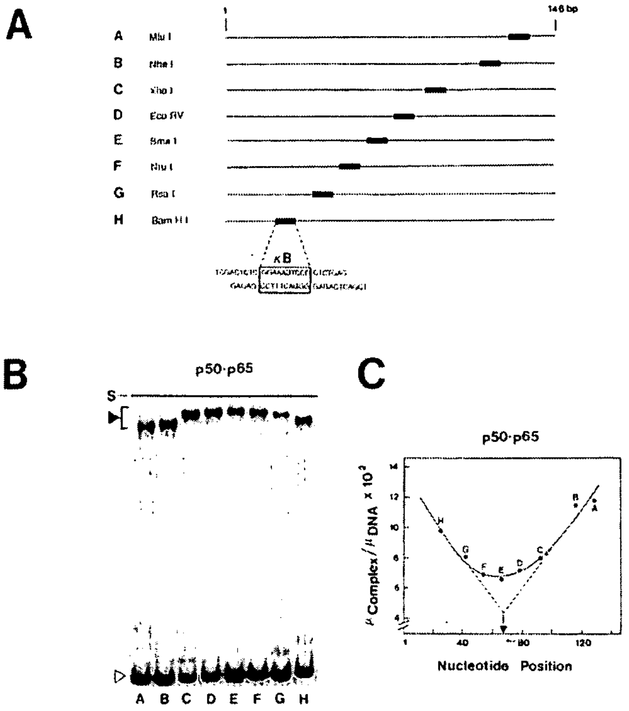

Fig.3. Circular permutation mobility shift assay with heterotetrameric NF-κB. (A) Eight 146 bp long fragments (A to H) were isolated from pUC-κB-Bend by cleavage with the indicated restriction endonucleases. The sequence of the inserted double-stranded oligonucleotide is shown with the decameric NF-κB binding site boxed. (B) The ^{32}P-endlabeled DNA fragments were incubated with heterotetrameric NF-κB and subjected to electrophoresis on a native 8% polyacrylamide gel. A fluorogram is shown. The solid line on top marks the starting position of the samples. The filled arrowhead and the bracket indicate the positions of NF-κB-DNA complexes of various mobilities, the open arrowhead the position of unbound DNA fragments. (C) The ratios of mobilities of NF-κB-DNA complexes (μ Complex) to those of the unbound fragments (μ DNA) is plotted against the position of the center of the NF-κB binding site within the 146 bp fragments. Dashed lines are an extrapolation of the flanks of the curve (22), and the filled arrowhead indicates the center of bending. The open arrowhead indicates the position of a hypothetical bending center which is obtained if bending would occur in the middle of the κB motif.

effect of the position of the binding site on the mobility of the protein-DNA complex was evident (Fig. 3B). Fragments with the binding site in the middle formed complexes with NF-κB which migrated much slower than complexes containing fragments where the κB site was located closer to either end. The mobilities of fragments that bind NF-κB in the middle (E) or at the end (A) showed an almost two-fold difference indicating a strong alteration of DNA structure. When the DNA fragments were not bound to the protein they all showed a very similar mobility in the 8% polyacrylamide gel used (Fig. 2B). This result suggests that the permutated DNA fragments did not differ in their presumably linear structure and that the κB motif displayed no anomalous DNA structure on its own.

The specificity of NF-κB binding was tested in a competition analysis (data not shown). A 50-fold molar excess of an unlabeled double-stranded 30 bp oligonucleotide containing an homologous κB motif abolished the radioactive complex formation while the same amount of a 30 bp oligonucleotide with the mutated κB motif 5'-GGGAATCTAA-3' had no significant effect. A radioactively labeled 120 bp XhoI fragment from the pUC-Bend vector with no inserted κB oligonucleotide was not detectably bound by NF-κB.

A quantitation of the results from the CP-EMSA is shown in Figure 3C. The relative mobilities of NF-κB-DNA complexes were plotted against the position of the center of the decameric binding site within fragments A to H. When the center of bending was determined by extrapolating the flanks of the curve (22,25) it became evident that the center of bending did not match with the center of the κB motif but was located at its 3'-end. This position overlaps or even coincides with the site where a nick in the DNA promotes binding of NF-κB (see Fig. 2). The data suggest that the heterotetrameric form of NF-κB containing the non-DNA-binding 65-kD subunit alters the DNA asymmetrically with respect to the center of the κB binding motif.

We have tested in CP-EMSAs the influence of various physical parameters on the mobility differences induced by NF-κB. To distinguish DNA bending from an increased flexibility of DNA, which can be caused by protein-induced melting or untwisting of DNA (26), we investigated the temperature dependence of the effects seen in CP-EMSAs. The mobility difference seen with fragments A and E at 4°C (Fig. 3) was reduced by only 6% if the gel was run at 37°C (Tab. 1) demonstrating that NF-κB alters the DNA structure also at a physiological temperature. The weak sensitivity towards increasing the temperature is consistent with bending of DNA. If NF-κB would partially melt the DNA, an increased temperature should augment rather than decrease the difference in mobility between complexes formed with circularly permutated DNA fragments. Alterations of the run length and

Table 1. Results from circular permutation mobility assays. (A) Five assays under different conditions were performed with purified heterotetrameric NF-$\varkappa$B. The changed parameters are underlined. Experiment 1 is shown in Figure 3B. Bending angles were estimated according to Thompson and Landy (28). (B) Two independent experiments were performed with NF-$\varkappa$B consisting solely of p50 homodimers. Experiment 6 is shown in Figure 4A.

	Experiment No.	Run Length (cm)	Temperature (°C)	Voltage, (V/cm)	Polyacrylamide Concentration of Gel (%)	Relative Mobility (μ_M/μ_E)	Bending Angle α
A	1	18	4	9	8	0.550	113.3
	2	38	4	9	8	0.600	106.0
	3	18	37	9	8	0.551	113.1
	4	18	4	4	8	0.611	105.0
	5	18	4	9	4	0.919	46.5
B	6	18	4	9	8	0.793	75.1
	7	18	4	9	8	0.798	74.1

the electric field had no or only a minor influence (Tab. 1). The only parameter that substantially reduced the ratio of mobilities was a lower polyacrylamide concentration of the gel. Presumably, the larger pore size decreased the resolution of the gel for differences in the shape of the various protein-DNA complexes.

Distamycin, a drug binding to the minor groove (27), was incubated with the DNA prior to addition of heterotetrameric NF-$\varkappa$B. It did not interfere at concentrations between 2.5 pM and 2.5 μM with the effect of spermine or the formation of a NF-$\varkappa$B-DNA complex (data not shown). The drug also did not alter the mobilities of complexes formed by NF-$\varkappa$B with the circularly permutated DNA fragments. This indicates that minor groove contacts of NF-$\varkappa$B are irrelevant for high affinity binding and DNA bending.

p65 can modulate the extent and position of DNA bending

The experiments described so far were performed with the heterotetrameric form of NF-$\varkappa$B which contains in addition to two DNA-binding p50 molecules also two non-DNA-binding p65 molecules. We have in the following investigated the influence of the p65 subunit on the various effects of NF-$\varkappa$B in CP-EMSAs by using a form of NF-$\varkappa$B which is devoid of p65 (7). In the absence of p65, the p50 DNA-binding subunit can form a dimer in solution and is bound to DNA as a dimer. As is characteristic for many dimerized DNA-binding proteins, p50 can bind with high affinity to completely palindromic motifs (9).

We have tested in CP-EMSAs whether the asymmetric alteration of the $\varkappa$B motif found for the heterotetrameric form of NF-$\varkappa$B depends on the presence of p65. p50 dimer was obtained by renaturing p50 which was separated from p65 on an SDS-polyacrylamide gel (7,9). Binding of the p50 dimer to the circularly permutated DNA fragments resulted in significantly smaller mobility differences than seen after binding of the heterotetramer. The mobility difference obtained with fragments that bind the p50 dimer in the middle or at the end was at most 1.25-fold (Fig. 4; Tab.1), as opposed to almost two-fold in the presence of p65 (Fig. 3). The bending angles (α) induced by the heterotetramer and p50 dimer were estimated by the empirical equation $\mu_M/\mu_E = \cos\alpha/2$ (28) from the ratio of the relative mobilities of complexes (μ) containing DNA fragments with the binding site in the middle (M) or at the end (E) (Tab.1). According to this approximation, the heterotetramer induced a bending angle in the order of 110° and the p50 dimer an angle of only 75° (Tab.1). This shows that p65 increased the bending angle induced by p50.

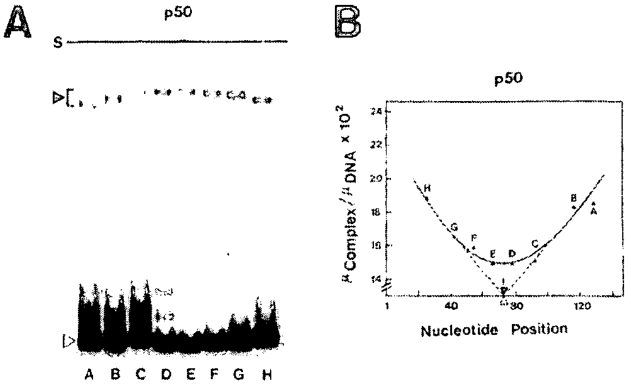

Fig.4. Circular permutation mobility assay with homodimeric p50 NF-$\varkappa$B. For details of the illustration see legend to Figure 3. (A) Purified homodimeric NF-$\varkappa$B which was prepared as described earlier (7) was reacted with the eight circularly permutated DNA fragments followed by analysis of protein-DNA complexes in native gels. A fluorogram is shown. (B) Quantitation of the results and determination of the bending center. Note that the scale on the ordinate is distinct from that of Figure 3C.

Also the bending center obtained with the p50 dimer was distinct from that seen with the heterotetramer. It was located precisely between fragments D and E and thus overlapped with a position corresponding to the center of the $\varkappa$B motif (Fig. 4B). Apparently, the p50 dimer makes a more even contact with the binding motif than the heterotetramer, resulting in a more symmetric bending of the $\varkappa$B DNA motif. From these results we conclude that the non-DNA binding 65-kD subunit of NF-$\varkappa$B can also alter the site of bending.

DISCUSSION

This study provides evidence that binding of the NF-$\varkappa$B transcription factor alters the structure of DNA. The alteration is most likely a bend or kink of the otherwise linear target DNA which is located within or close by the decameric binding site for NF-$\varkappa$B depending on the association of the p50 dimer with the 65-kD subunit. DNA bending was evident from the effect that the position of the protein binding site within DNA fragments of identical length influenced the mobility of the NF-$\varkappa$B-DNA complexes. The relative temperature-independence of the mobility shifts indicated that the change in DNA structure induced by the transcription factor was a bending of DNA rather than a local increase in its flexibility. This is supported by the observation that the binding of NF-$\varkappa$B to DNA is facilitated in the presence of various agents known to promote and stabilize DNA bending.

The structural alteration induced by the p50 dimer could be a bend which stretches evenly over the entire decameric binding sequence or a kink which is localized in the center of the motif. It is also possible that two kinks induced by each of the two p50 molecules add up their angles depending on their distance to each other. In case of the heterotetramer, an additional kink at the 3′-end of the binding sequence could be induced which adds up its angle to that of a weaker central kink or to that of a kink located in the 5′-half of the motif. Alternatively, a bend or kink is shifted from the center towards the 3′-end of the motif and its angle is increased by the action of p65 onto p50.

The experiments shown in Figures 1 and 2 suggest that binding of NF-$\varkappa$B to linear DNA is energetically less favorable than binding to nicked or bent DNA, presumably because the electrostatic repulsion of phosphate groups in the DNA backbone poses an energy barrier upon bending. When negative charges are neutralized by bound spermine or metallic cations, or the flexibility of the DNA is increased by a nick in the backbone, the energy requirement for bending is lowered and, as a consequence, binding of NF-κB to DNA is enhanced. By analogy with the DNA bend induced by the CAP protein (29), we assume that the NF-κB-induced DNA-bending requires and the bend subsequently stores free energy. We recently demonstrated that the inhibitory subunit of NF-κB, IκB, can effectively increase the dissociation rate of a high-affinity NF-κB-DNA complex (8). This involved the dissociation of protein-protein interactions in the DNA-bound heterotetramer by IκB. The velocity of such a reaction might be greatly enhanced by releasing the energy that is stored in the protein-induced bend.

The DNA-binding motifs of two other transcription factors that induce bending, CBF/SRF (30) and the papilloma virus type 1 protein E2 (31), show a striking similarity to that of NF-κB. CBF/SRF binds to the consensus sequence CC(A+T-rich)$_6$GG and the E2 protein to ACCN$_6$GGT. The consensus of κB sites can be written as GG(A+T-rich)$_6$CC or GGN$_6$CC and thus differs from the two other sites only in the symmetry but not in the spacing of G and C clusters. (The p50 dimer can bind with high affinity to the κB motif 5′-GGAAATTTCC-3′ (9).) The consensus motif CNNG(A+T-rich)$_6$CNNG for the bend-inducing heat shock transcription factor (32) seems to be more distantly related but again an A-T-rich central sequence of six nucleotides is flanked by conserved C and G residues in a palindromic arrangement suggesting that bend-inducing DNA-binding proteins have related cognate motifs.

The significance of DNA-bending for stimulation of transcription is unclear. In eukaryotic genes, bent DNA segments might act as cis-acting elements, as it has been demonstrated in prokaryotes (10,11). For instance, in the *gal* promoter of *E. coli* an intrinsically bent DNA fragment can functionally substitute for the binding site of the DNA-bending CAP transcription factor. Swap experiments in which NF-κB binding motifs are replaced by intrinsically bent DNA fragments and experiments investigating the correlations between the bending angle induced in a κB motif, the affinity of a motif and its cis-acting potential would allow to test the effect of DNA bends on transcriptional initiation in eukaryotes.

Most of the currently identified NF-κB binding sites are of reduced symmetry and consist of pentameric half sites which are distinct in their degree of sequence conservation (15). The more conserved half site A has the consensus 5′-GGGPuN-3′ while the second half site B tolerates a much broader variation of sequence. In the κB motif 5′-GGGACTTTCC-3′, which is most frequently found in enhancer elements, half site A appears to be recognized with higher affinity by the protein than half site B, as suggested by competition experiments using isolated κB half sites within identical flanking sequences. The relationship between the symmetry of NF-κB binding motifs and the affinity of binding, and the role of the p65 subunit for the sequence recognition by p50 was investigated in a subsequent study (8). It was shown that, if p50 is associated with the p65 subunit, the physiological κB motif 5′-GGGACTTTCC-3′ is bound with a two-fold higher affinity whereas completely palindromic motifs are recognized with a more than ten-fold lower affinity by p50. The data suggested that the interaction of p50 with DNA is altered by the non-DNA binding 65-kD subunit of NF-κB. The effect of p65 on the p50-induced bending observed in the present study parallels the effect of p65 on the DNA sequence recognition by p50: p65 seems to cause an unequal interaction of the p50 molecules with the decameric cognate sequence. In one case, this is evident from the shift of the bending center towards the 3′-end of the motif, in the other case, by the preference of the heterotetramer for binding motifs of reduced symmetry and half sites of distinct sequence conservation. It is likely that these observations are directly related.

We assume that each half site of the κB motif is bound by one p50 molecule in a head to head orientation. The p50 molecules in the NF-κB homodimer appear to interact equally with the half sites of the κB motif 5′-GGGACTTTCC-3′ with respect to the induction of DNA bending although the half sites are recognized with a slightly distinct affinity by the p50 molecules (9). The p50 molecules in the NF-κB heterotetramer recognize the half sites of the κB motif not only with different affinity but, in contrast to the dimer, the p50 molecules appear to subject their half sites differently to a structural modification. This raises the possibility that the two p50 molecules in the heterotetramer are modified by the p65 molecules such that they received distinct DNA-binding and -bending properties. It is however hard to envision how two indistinguishable p65 molecules in the heterotetramer can give raise to two distinct p50 molecules. An alternative possibility is that both p50 molecules are still functionally equivalent but are altered in their quarterny structure by p65. We indeed have evidence that the p50 molecules in the heterotetramer are differently linked than in the dimer (M.B. Urban and P.A. Baeuerle, in preparation). An alternative determinant for their differential interaction with the half sites of the κB motif would than be the distinct DNA sequences of the half sites. Half site B might preferentially undergo bending because it has a higher content of A and T bases. Certain A-T rich DNA stretches from kinetoplast DNA were shown to bend even in the absence of proteins (33). One p50 molecule would bind the more conserved 5′-half site A with high affinity, which serves to stabilize the protein-DNA interaction, while the second p50 molecule binds more weakly to the less conserved 3′-half site B but can alter its helical structure. Further studies are required to investigate the sequence of events leading to NF-κB-DNA recognition and to obtain a more highly ressolved picture of the DNA structure induced by p50 in the absence and presence of the p65 subunit.

MATERIALS AND METHODS

Oligonucleotides and vectors

Oligonucleotides were synthesized on an Applied Biosystems synthesizer and purified on cartridges (Applied Biosystems) according to the instructions provided by the manufacturer.

An EcoRI-HindIII fragment from the pBend2 vector (23) was cloned into an EcoRI-HindIII cleaved pUC9 vector (34). pUC-κB-Bend resulted from the insertion of a 30 bp oligonucleotide (Fig. 3A) with SalI linker sites into the SalI opened pUC9-Bend. Fragments of identical length were excised from the plasmid by the indicated restriction endonucleases (all purchased from Biolabs).

Electrophoretic mobility shift assays

The conditions for the standard EMSA are described elsewhere (15,35). The oligonucleotide used as DNA probe in Fig. 1 was labeled with α-[^{32}P]-dCTP by the Klenow polymerase (Boehringer).

Modifications of EMSA conditions that were applied with the circularly permutated DNA fragments are indicated in Table 1. Phosphatase-treated circularly permutated DNA fragments were end-labeled with polynucleotide kinase (Biolabs) as described (36) and purified on an polyacrylamide gel followed by electroelution. For one assay approximately 10,000 c.p.m. (Cerenkov counting; 4.5 fmoles) were used. Two hundred nanograms of poly(dI-dC) (Pharmacia) were included per assay. The NF-κB proteins were purified as described earlier (7,8,15).

Hydroxyl radical missing contact analysis

A double-stranded 30 bp oligonucleotide with the NF-κB site 5′-GGGACTTTCC-3′ and flanking sequences from the mouse κ light chain enhancer (sequence in ref. 15) was cloned into the HindIII and SalI sites of the Bluescript minus vector (Stratagene) and ^{32}P-labeled at the 5′-end by a strategy analogous to that described previously (37). Hydroxyl radical treatment of the labeled DNA was as follows: 650 fmoles of DNA in 25 mM HEPES-KOH, pH 7.5, and 150 mM NaCl were incubated for various times with 10 mM ascorbic acid, 10 μM $(NH_4)_2Fe(SO_4)_2$, 20 mM EDTA and 0.3% H_2O_2. Reactions were stopped by the addition of 10 mM thiourea, 2 mM EDTA and DNA was precipitated with linear polyacrylamide. The extent of the reaction was determined by electrophoretic analysis. The sample with a ratio of reacted to unreacted DNA of 3:10 was used for EMSA.

ACKNOWLEDGEMENTS

We are indebted to Susi Kunz for excellent technical assistance, Dr. Georg Arnold and Andrea Oswald for synthesis of oligonucleotides and Mark Malia and Drs. Hermann Heumann, Lienhart Schmitz, Prof. Dr. Günther Gerisch and the reviewers for helpful comments on the manuscript. This work was supported by a grant from the Bundesministerium für Forschung und Technologie and is part of the doctoral thesis of R.S.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 266, No. 1, Issue of January 5, pp. 252-260, 1991
Printed in U.S.A.

DNA Binding of Purified Transcription Factor NF-κB

AFFINITY, SPECIFICITY, Zn^{2+} DEPENDENCE, AND DIFFERENTIAL HALF-SITE RECOGNITION*

(Received for publication, April 19, 1990)

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A rapid purification procedure for the NF-κB transcription factor from the cytosol of human placenta is demonstrated which exploits the insensitivity of the NF-κB·DNA complex toward the intercalating agent chloroquine. Purified NF-κB required 100 mM KCl or NaCl and a pH of 7.5 to optimally bind to DNA. Equilibrium of binding was reached within less than 5 min in the absence of competitor DNA and after 1 h in the presence of 0.1 mg/ml poly(dI-dC). DNA binding of NF-κB was specifically blocked by the chelating agent 1,10-orthophenantroline and could only be reconstituted by addition of Zn^{2+}. Under optimal binding conditions, the dissociation constant for the complex of the purified NF-κB with its most frequent cognate DNA motif 5′-GGGACTTTCC-3′ was in the range of 10^{-12} to 10^{-13} M. Various other cis-acting κB motifs were recognized by NF-κB with lower affinities. A comparative analysis of known NF-κB-binding sites and competition experiments with synthetic polynucleotides and oligonucleotides encompassing only one half-site or single-stranded κB motifs suggested that the two DNA-binding monomers in the NF-κB protein complex can interact differentially with the half-sites of the decameric cognate motif.

The NF-κB transcription factor is a sequence-specific DNA-binding protein (Sen and Baltimore, 1986a) that initiates transcription (Kawakami *et al.*, 1988) from a variety of genes that are all involved in immune response, acute phase, and inflammatory processes (for reviews, see Lenardo and Baltimore, 1989; Baeuerle and Baltimore, 1991). The active form of NF-κB is a heterotetramer which contains two 50-kDa DNA-binding polypeptides (p50) (Kawakami *et al.*, 1989) to which two 65-kDa non-DNA-binding polypeptides (p65) are attached (Baeuerle and Baltimore, 1989). Only dimerized p50 appears to bind with high affinity to DNA. Apparently uninfluenced by p65, p50 recognizes the decameric sequence 5′-GGGACTTTCC-3′ and variations thereof.

In most cells, NF-κB resides in a latent form in the cytoplasm which can be activated *in vivo* by a great variety of agents including cytokines, viral transactivator proteins, and activators of protein kinases (Baeuerle and Baltimore, 1988a, 1988b; for reviews see Lenardo and Baltimore, 1989; Baeuerle and Baltimore, 1991). *In vitro*, NF-κB can be activated by treatment of cytosolic fractions with sodium deoxycholate, formamide, or by electrophoretic size fractionation (Baeuerle and Baltimore, 1988a). The inducible form of NF-κB is stabilized by a specific inhibitory subunit of NF-κB, called IκB (Baeuerle and Baltimore, 1988b). In complex with IκB, NF-κB is a heterotrimer composed of IκB-α (37 kDa) or IκB-β (43 kDa), p50 and p65 (Zabel and Baeuerle, 1990). The IκB variants presumably keep NF-κB inactivated by preventing the dimerization of the DNA binding p50. NF-κB can be inhibited *in vitro* by IκB only if p65 is bound to p50 (Baeuerle and Baltimore, 1989). Recent data from our laboratory suggest that one function of the p65 subunit in the inducible form of NF-κB is to serve as receptor for IκB[1] (Urban and Baeuerle, 1990). In the active nuclear form of NF-κB, p65 serves as a modulator of the DNA binding specificity and is perhaps involved in transcription initiation.

There is evidence that IκB is inactivated and subsequently dissociated from NF-κB by its phosphorylation through protein kinase C[1] (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990). Following dissociation of IκB and association of two p50-p65 heterodimers, NF-κB can enter the nucleus and activate genes upon binding to various κB motifs. In that way, NF-κB actively participates in cytoplasmic nuclear signaling (Baeuerle and Baltimore, 1988b).

The predominant form of active NF-κB from nuclear extracts and deoxycholate-treated cytosol is heterotetrameric as indicated by the mobility of protein/DNA complexes in EMSA[2] (Baeuerle and Baltimore, 1989). We therefore studied the DNA binding activity of the form of NF-κB which contains both the p50 and p65 proteins. We report here on a rapid purification procedure for NF-κB, the kinetics of DNA binding, a Scatchard analysis of the DNA binding affinity, and on the ion, pH, and temperature requirements for optimal DNA binding. Data from competition experiments with synthetic polynucleotides and various oligonucleotides encompassing physiological κB motifs, κB half-sites, and single-strands from κB motifs as well as a comparative analysis of known NF-κB-binding sites suggest that the two p50 subunits in the NF-κB complex can interact differentially with the half-sites of decameric cognate motifs. Our study might help to distinguish other DNA-binding proteins that can also interact with κB motifs from NF-κB.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—DEAE-Sephacel and Cm-Sepharose CL-6B were purchased from Pharmacia LKB Biotechnology Inc. and the DNA affinity resin (multimerized κB DNA motifs attached to cyanogen bromide-activated Sepharose 4B) was prepared as described

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* This study was supported by the Bundesministerium für Forschung und Technologie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ This work was done in partial fulfillment of a doctoral thesis.

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[1] U. Zabel and P. Baeuerle, unpublished data.

[2] The abbreviations used are: EMSA, electrophoretic mobility shift assay; IL-2, interleukin-2; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

(Baeuerle and Baltimore, 1989). Chloroquine, Nonidet P-40, cyanogen bromide-activated Sepharose 4B, 1,10-orthophenanthroline, and bovine serum albumin (No. 7409) were supplied by Sigma. The synthetic polynucleotides poly(dA-dT) and poly(dG-dC) were purchased from Boehringer Mannheim and poly(dI-dC) from Pharmacia.

Oligonucleotides and Labeling—Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems Synthesizer followed by purification on oligonucleotide purification cartridges (Applied Biosystems) according to the instructions provided by the manufacturer. All double-stranded oligonucleotides were 30-mers with *Hin*dIII and *Sal*I linker sites on the 5′- and 3′-ends, respectively, which were annealed from 26-mers. The sequence of the "κB" oligonucleotide used as radioactive DNA probe was as follows:

5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′
3′-AGTCTCCCCTGAAAGGCTCTCCAGCT-5′

The oligonucleotides used for competition had identical length and linker sites. Their decameric κB motifs were also placed in the center of the DNA fragments and had five base pairs of the flanking sequences found *in situ*. For use in EMSAs, the double-stranded κB oligonucleotide was labeled with [α-^{32}P]dCTP (Amersham Corp., 3,000 Ci/mmol) by the Klenow polymerase (Boehringer Mannheim) and gel-purified on low melting agarose gels as described (Schreck and Baeuerle, 1990).

Affinity Purification of NF-κB—The initial purification steps by conventional procedures were described in detail elsewhere (Zabel and Baeuerle, 1990). Briefly, one human placenta was homogenized in hypotonic buffer and proteins in the high speed supernatant precipitated in 40% saturated ammonium sulfate. Dialyzed fractions were subjected to anion-exchange chromatography on DEAE-Sephacel. Fractions containing NF-κB·IκB complex were pooled, dialyzed, and subjected to cation-exchange chromatography on Cm-Sepharose CL-6B at pH 5.5. This treatment induced dissociation of the NF-κB·IκB complex and separation of activated heterotetrameric NF-κB and IκB into distinct column fractions. The further purification of IκB variants is described elsewhere (Zabel and Baeuerle, 1990). The fractions containing activated NF-κB were pooled and diluted by one volume of buffer H (20 mM HEPES, pH 7.9, 20% glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, 0.25 mM EGTA, 0.2% Nonidet P-40, 0.05% sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride) to a final concentration of approximately 100 mM KCl. The pH was adjusted to 7.5 by the addition of 1 M Tris-HCl, pH 8.0. At a flow rate of 50 ml/h, the solution was passed twice over 2.5 ml (wet packed resin) of a DNA affinity resin (Baeuerle and Baltimore, 1989). The column was washed with 50 ml of buffer H containing 100 mM KCl and 5 mM chloroquine. A second wash was with 10 ml of buffer H containing 200 mM KCl and 5 mM chloroquine and a third wash used buffer H with 200 mM KCl and no chloroquine. NF-κB was eluted with 5 ml of buffer H containing 300 mM KCl.

Electrophoretic Mobility Shift Assays—The standard DNA-binding reaction was performed at room temperature in a volume of 20 μl and contained 1 mg/ml bovine serum albumin, binding buffer (Sen and Baltimore, 1986), 5–10,000 cpm (Cerenkov counting) ^{32}P-labeled κB DNA probe (approximately 0.1 ng of DNA), 0.1 mg/ml poly(dI-dC), 2 μl of buffer D (Dignam *et al.*, 1983) with 1% (v/v) Nonidet P-40, and 0.5–2 μl of NF-κB in buffer H containing 300 mM KCl. After the indicated incubation times, samples were subjected to electrophoresis on native 4% polyacrylamide gels as described (Sen and Baltimore, 1986). The standard binding reaction was modified for the experiments shown in Figs. 2, 4, and 5 (see figure legends). To determine the effects of various NaCl and KCl concentrations (Fig. 2, *A* and *B*), a binding buffer without NaCl was used. The pH optimum of DNA binding (Fig. 2*C*) was determined by using an unbuffered binding solution to which 100 mM buffer solutions of HEPES-NaOH and Tris-HCl with various pH values were added. The DNA binding activity of NF-κB was determined by Cerenkov counting of pieces from the dried native gel containing the ^{32}P-labeled NF-κB·DNA complex.

Determination of the Dissociation Constant—The K_D value was determined using EMSAs as described (Meisterernst *et al.*, 1988). A double-stranded oligonucleotide was used which contained in its center the κB motif from the mouse κ light chain enhancer (Sen and Baltimore, 1986) and 4 base pairs which flank the site *in situ*. Each 5′-end of the oligonucleotide contained a stretch of 10 thymidine residues which allowed to fill in a maximum of 20 α-^{32}P-labeled adenosine residues of known specific activity/oligonucleotide. Two cytosine residues at each 5′-end were filled in with an excess of unlabeled dGTP to prevent the loss of labeled adenosine residues from the oligonucleotide by a back reaction. By using high amounts of the radioactive DNA probe in EMSAs, the number of DNA-binding sites in the NF-κB solution was determined. The DNA-binding reactions were performed in the absence of poly(dI-dC), the presence of 100 mM NaCl, and for 20 min at room temperature followed by EMSA analysis. The radioactivity in one complete gel lane separated into complexed and free DNA was determined by scintillation counting and numbers corrected for counting efficiency and decay of ^{32}P.

1,10-Orthophenantroline Treatment—A 100 mM solution of 1,10-orthophenantroline was made up in ethanol and diluted to 20 mM by the addition of 4 volumes of double-distilled H$_2$O. With 1–2 μl of this solution, NF-κB was reacted in 20 μl of a standard DNA-binding reaction in the absence of DNA. Control samples obtained the same volume of 20% ethanol. After 20 min, the DNA probe was added and DNA binding allowed for 30 min. The standard DNA-binding reaction contained already 0.5 mM EDTA and no divalent cations.

RESULTS

High Efficiency DNA Affinity Purification of NF-κB by the Use of Chloroquine—In previous attempts, numerous chromatography steps were required to isolate NF-κB from nuclear extracts of B lymphocytes (Kawakami *et al.*, 1988; Lenardo *et al.*, 1988), and at least four rounds of DNA affinity chromatography in the presence of the costly competitor poly(dI-dC) had to be used to purify NF-κB from the cytosol of HeLa cells (Baeuerle and Baltimore, 1989). We noticed that the presence of up to 20 mM chloroquine in DNA-binding reactions had no detectable influence on the stability of the protein-DNA complex formed by NF-κB (data not shown). In the absence of high concentrations of poly(dL-dC), no single step affinity purification can be achieved (see Fig. 1*D*; Baeuerle and Baltimore, 1989), and even multiple rounds of DNA affinity chromatography were ineffective (data not shown).

We noticed that the presence of up to 5 mM chloroquine in DNA-binding reactions had no detectable influence on the stability of a preformed protein-DNA complex of NF-κB (Fig. 1*A*, *lanes 2–8*). If the DNA probe is preincubated with 5 mM chloroquine, only a small reduction of NF-κB binding is seen compared with a sample in which a preformed protein-DNA complex was incubated with 5 mM chloroquine (Fig. 1*A*, compare *lanes* 9 and *10*). In contrast, other sequence-specific DNA-binding proteins, such as the serum response factor, were shown to be highly sensitive toward the intercalating activity of chloroquine (Schröter *et al.*, 1987). Here we subjected the NF-κB which was activated during the purification of IκB (Zabel and Baeuerle, 1990) to sequence-specific DNA affinity chromatography and examined whether chloroquine in washing buffers can increase the efficiency of purification of NF-κB by selectively removing contaminating unrelated DNA-binding proteins from the DNA resin.

A dramatic purification effect was indeed evident when washing buffers containing 5 mM chloroquine were used (Fig. 1*B*). In a 200 mM KCl wash, which followed the second washing step with 5 mM chloroquine at 200 mM KCl, barely any proteins were detectable in silver-stained SDS-gels (Fig. 1*B*, *lane 5*). Following elution with 300 mM KCl, approximately 10% of the applied NF-κB DNA binding activity could be recovered as determined by EMSA (Table I; Fig. 1*C*, *lane 6*). At 400 mM KCl, another 10% of the NF-κB activity eluted but it contained more protein contaminants (data not shown). No activity was detected in a wash with 1 M KCl. Among the few polypeptides that eluted at 300 mM KCl from the column, p50 and p65 were the major species (Fig. 1*B*, *lane 6*). These two polypeptides were not seen in the preceding wash which showed almost no NF-κB activity (Fig. 1*B*, *lane 5*).

Although the low amounts of protein in the affinity eluate could not be determined, an enormous purification factor by the DNA affinity chromatography becomes apparent if the

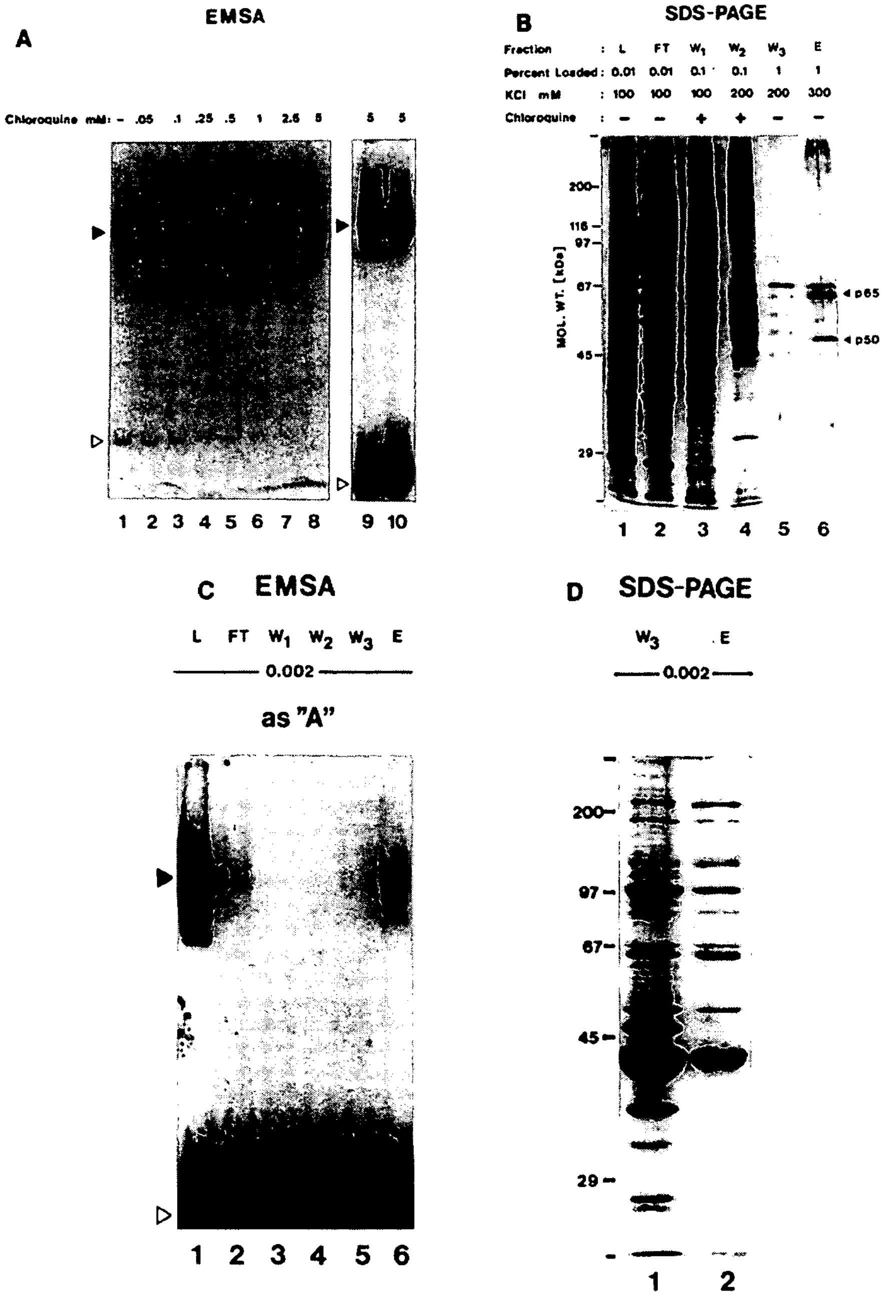
A
EMSA
Chloroquine mM: – .05 .1 .25 .5 1 2.5 5 5 5
1 2 3 4 5 6 7 8 9 10
B
SDS-PAGE
Fraction : L FT W1 W2 W3 E
Percent Loaded : 0.01 0.01 0.1 0.1 1 1
KCl mM : 100 100 100 200 200 300
Chloroquine : – – + + – –
MOL. WT. [kDa]
200
116
97
67
45
29
p65
p50
1 2 3 4 5 6
C EMSA
L FT W1 W2 W3 E
0.002
as "A"
1 2 3 4 5 6
D SDS-PAGE
W3 E
0.002
200
97
67
45
29
1 2

FIG. 1. **DNA affinity purification of NF-κB.** *Panel A*, insensitivity of NF-κB toward chloroquine. Purified NF-κB was incubated with a ^{32}P-labeled κ enhancer DNA probe and the indicated amounts of chloroquine added after 10 min. After another 10 min, samples were analyzed by EMSA on native gels (*lanes 1-8*). In *lane 10*, the DNA probe was preincubated for 10 min with 5 mM chloroquine. 10 min after addition of NF-κB the sample was analyzed by EMSA. *Lane 9*, control in which chloroquine was added last. *Panel B*, SDS-polyacrylamide gel electrophoresis analysis of column fractions. The indicated percentage of fractions obtained during DNA affinity purification of NF-κB were precipitated by 80% acetone and subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions (Lämmli, 1970). The gels were stained with silver by the method of Heukeshoven and Dernick (1988). *L*, load; *FT*, flow through; *W*, wash; *E*, eluate. The KCl concentration of buffers and the presence of 5 mM chloroquine (+) is indicated. Molecular size markers were myosin (200 kDa), galactosidase b (116 kDa), phosphorylase (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and carboanhydrase (29 kDa). The positions of p50 and p65 proteins are indicated by *arrowheads*. *Panel C*, analysis of 0.002% of the column fractions for the DNA binding activity of NF-κB in EMSAs. *Panels A* and *C* show fluorograms from 4% native polyacrylamide gels. *Filled arrowhead*, position of the NF-κB-DNA complex; *open arrowhead*, position of unbound DNA. *Panel D*, DNA affinity purification in the absence of 5 mM chloroquine. A Coomassie Blue-stained 10% SDS-gel is shown. The purification was performed as described in *panel B* except that 5 mM chloroquine was added to the wash buffers. Only the third wash (W_3) and the 300 mM KCl eluate are shown.

TABLE I

Purification of NF-κB from the cytosol of human placenta

The DNA binding activity of NF-κB was determined by EMSA under standard assay conditions (see "Experimental Procedures"). All fractions were assayed in the same experiment and in the presence of sodium deoxycholate as described (Baeuerle and Baltimore, 1988a). The protein-DNA complexes detected on fluorograms were quantitated by Cerenkov counting. Protein concentrations were determined by Coomassie Blue binding (Biorad Microassay). ND, not determined.

Fraction	Total protein	Total NF-κB activity	Yield	Specific activity	Purification
	mg	*cpm × 10^{-6}*	*%*	*cpm × 10^{-6} × mg^{-1}*	*-fold*
Cytosol	3,760	314	100	0.084	1
Precipitation by 40% ammonium sulfate	408	129	41	0.316	3.8
DEAE-Sephacel	42.3	67	21	1.584	5
CM-Sepharose	11.0	61	19	5.545	3.5
DNA affinity	ND	6.9	2.2	ND	ND

protein pattern from *lane 1* (load, Fig. 1*B*) is compared to that of *lane 6* (eluate, Fig. 1*B*). In the latter, a hundred times more of the fraction was loaded. We estimate that *lane 6* contained less than 1% of the amount of protein seen in *lane 1*. Considering the yield of 10% (Table I), we assume that the purification factor achieved by the affinity step was in the range of 1,000–5,000-fold. As outlined in Table I, highly purified NF-κB can be obtained from the cytosol of human placenta by a simple and rapid procedure. The overall purification factor of NF-κB in the purification shown was presumably in the order of 100,000-fold. Prolonged washing with the buffer containing 200 mM KCl (Fig. 1*B*, *lane 5*) allowed to remove most of the minor protein contaminants seen in the 300 mM KCl eluate (data not shown).

The Effects of Ions, pH, and Temperature on the DNA Binding Activity of NF-κB—To evaluate the effects of sodium and potassium chloride on DNA binding, we used a small amount of NF-κB that gives only a weak signal under the standard assay condition using 50 mM NaCl (Fig. 2*A*, *fourth lane*). At concentrations of NaCl and KCl below 50 mM, no significant binding activity was detected (Fig. 2, *A* and *B*). Concentrations above 50 mM KCl or NaCl strongly increased binding of NF-κB to DNA. With both salts, optimal binding occurred at concentrations of approximately 100 mM suggesting that the effect was not dependent on the nature of the monovalent cation. The amounts of NF-κB·DNA complex formed at the optimal KCl and NaCl concentrations were comparable. These observations suggest that monovalent cations can positively influence the affinity of DNA binding and show that the binding conditions applied so far to assay for NF-κB (Sen and Baltimore, 1986) were suboptimal.

To test whether the pH 7.5 of the standard binding reaction allowed optimal DNA binding, we added HEPES and Tris-HCl buffer solutions of various pH values to binding reactions. NF-κB could form a complex with DNA within a pH-range from 6 to 8.5 and the highest amount of protein-DNA complex was formed at a physiological pH of 7.5 (Fig. 2*C*).

The effect of increased temperature on the stability and formation of a NF-κB·DNA complex was investigated. Temperatures above 45 °C irreversibly destroyed the activity of NF-κB (Fig. 2*D*). The binding activity at 37 °C was similar to that at room temperature and it made no difference whether the protein was heat treated in the absence of DNA (data not shown) or in a protein-DNA complex which was preformed at room temperature (Fig. 2*D*) indicating that the protein was not stabilized to heat denaturation in complex with DNA.

NF-κB Requires Zn^{2+} for DNA Binding—We have included in DNA-binding reactions 1,10-orthophenantroline, a chelating agent that is commonly used to study the zinc requirement of proteins (Falchuk *et al.*, 1976). Increasing concentrations of the chelator impaired the formation of a NF-κB·DNA complex (Fig. 3, *lanes 2–4*). 75% inhibition was observed at a

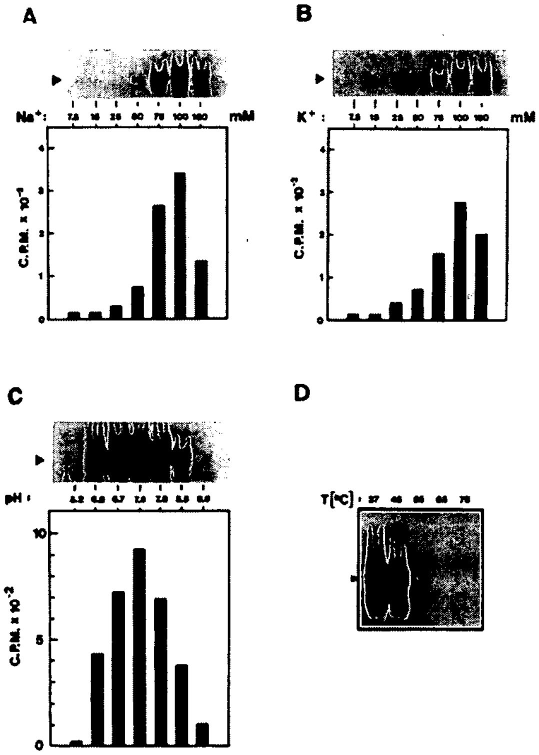

FIG. 2. **The effects of salt, pH, and temperature on the DNA binding of NF-κB.** *Panel A*, the effect of NaCl. *Panel B*, the effect of KCl. In a standard DNA-binding reaction (see "Experimental Procedures") a binding buffer without salt was used and NaCl or KCl solutions added to give the final salt concentrations indicated. All reactions had in addition 7.5 mM KCl which came from the elution buffer for NF-κB. *Panel C*, the effect of pH. A DNA-binding buffer was used which contained either 100 mM HEPES-NaOH, pH 5.2–7.5, or 100 mM Tris-HCl, pH 7.8–9. A Tris-HCl buffer with pH 7.5 gave similar results as the HEPES-NaOH buffer of pH 7.5 (data not shown). The buffer solutions were tested for compensation of the intrinsic pH contributed by the ingredients of the binding reaction mixture. On the top of *panels A–C*, sections of fluorograms are shown containing the NF-κB-DNA complex (*filled arrowhead*). The radioactivity in the complexes was determined by Cerenkov counting. *Panel D*, the effect of temperature. A complex of NF-κB and DNA was allowed to form for 20 min at room temperature and then exposed to various temperatures. After 5 min samples were chilled on ice and analyzed on 4% native gels at room temperature. A section of a fluorogram is shown.

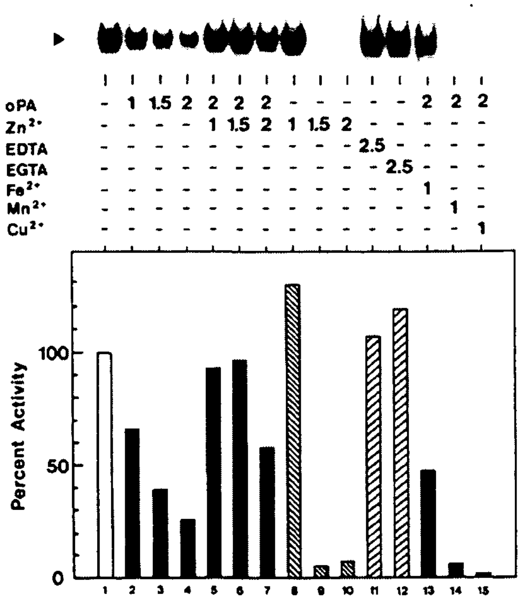

FIG. 3. **The effect of chelating agents on NF-κB.** Purified NF-κB was incubated in a standard DNA-binding reaction with the indicated concentrations (in mM) of agents. *oPA*, 1,10-orthophenantroline. After 20 min the radioactive DNA probe (*κB*) was added. The quantitation of the DNA binding activity is shown in the *bottom panel* and a section of a fluorogram in the *top panel*. *Open column*, control; *filled columns*, presence of 1,10-orthophenantroline; *shaded columns*, the effect of Zn^{2+} (*lanes 8-10*) and the effect of other chelators (*lanes 11* and *12*).

concentration of 2 mM 1,10-orthophenantroline (Fig. 3, *lane 4*). (Higher concentrations caused a precipitate in the samples.) At a concentration of 2 mM, the chelators EDTA and EGTA had no inhibiting effect (Fig. 3, *lanes 11* and *12*). The inhibition of DNA binding by 2 mM 1,10-orthophenantroline could be reversed by addition of only 1 or 1.5 mM Zn^{2+} (Fig. 3, *lanes 5* and *6*). The observation that zinc ions in the presence of excess chelator can fully reconstitute DNA binding suggests that the affinity of NF-κB for Zn^{2+} is much higher than that of 1,10-orthophenantroline. Unlike Zn^{2+}, other divalent cations at a concentration of 1 mM such as Fe^{2+} (Fig. 3, *lane 13*), Mn^{2+} (*lane 14*), and Cu^{2+} (*lane 15*) could not reconstitute the DNA binding activity of NF-κB following 1,10-orthophenantroline treatment. Mn^{2+} and Cu^{2+} ions were rather inhibiting. These data suggest that NF-κB specifically requires Zn^{2+} for optimal DNA binding. In the absence of 1,10-orthophenantroline, 1 mM Zn^{2+} slightly increased DNA binding (Fig. 3, *lane 8*) but, intriguingly, 1.5 and 2 mM Zn^{2+} were strongly inhibiting (*lanes 9* and *10*).

NF-κB DNA Binding in the Presence of Polynucleotides with Alternating Sequence—NF-κB and a labeled κB oligonucleotide were reacted for various times in the absence or presence of 0.1 mg/ml poly(dI-dC) and binding reactions stopped by electrophoretic separation of DNA from protein-DNA complexes. Without poly(dI-dC), the protein-DNA complex could form very rapidly. Within less than 5 min the binding reaction had approached an equilibrium (Fig. 4*A*) reflecting a high on-rate of protein binding to DNA. In the presence of a 10^6-fold weight excess of poly(dI-dC) over the DNA probe, the equilibrium of binding was reached after 1 h and only 20% of the amount of radioactive complex could form which was seen in the absence of the polynucleotide. Here we further used polynucleotides of various base composition in competition assays to characterize the DNA binding specificity of NF-κB.

Purified NF-κB could be assayed in EMSAs in the complete absence of a nonspecific competitor DNA (Fig. 4*B*, *last lane*). No additional binding activities were seen indicating that the NF-κB fraction used was essentially free of unrelated DNA-binding proteins. Addition of increasing amounts of polynucleotides showed that poly(dA-dT) had a much higher efficiency than poly(dI-dC) to compete for binding of NF-κB to the radioactive DNA probe (Fig. 4*B*). In Fig. 4*C*, we show the quantitation of titration experiments with three different polynucleotides. Poly(dI-dC) was the weakest competitor with an IC_{50} of 38 μg/ml. If a polynucleotide is used which has guanosine instead of inosine residues a 50-fold better competition was observed; the IC_{50} for poly(dG-dC) was only 0.75 μg/ml. The IC_{50} of poly(dA-dT) was 2.8 μg/ml. These data suggest that guanosine residues contribute most to the recognition of DNA by NF-κB which would be consistent with the high conservation of guanosine residues in all known κB motifs (see Fig. 6). While adenosine and/or thymidine residues also significantly contribute to the protein-DNA binding, cytosine residues appear to be least important.

High Affinity DNA Binding of NF-κB—We have determined the dissociation constant of a complex of NF-κB with the cognate κB motif 5′-GGGACTTTCC-3′ which is the most frequent NF-κB-binding site found in enhancer elements so far (see Fig. 6*A*). Results from competition experiments (Fig. 7, Schreck and Baeuerle, 1990) suggested that this site is recognized with the highest affinity by NF-κB. From another study it was apparent that the dissociation constant must be smaller than 10^{-11} M, and therefore its determination requires a DNA probe of very high specific activity (Baeuerle and Baltimore, 1989). Titration of NF-κB with increasing amounts of DNA probe showed a saturation of complex formation in EMSAs which allowed us to determine the number of DNA-binding sites of NF-κB in the preparation used (data not shown). By titration with decreasing amounts of the DNA probe and determination of the ^{32}P radioactivity in the positions of complexed and free DNA in native gels (Meisterernst *et al.*, 1988), we calculated by a Scatchard analysis a dissociation constant of the NF-κB-DNA complex of 2.7×10^{-12} M (Fig. 5). If NF-κB was used which was reconstituted from SDS-gel purified p50 and p65 proteins, a K_D value of 4×10^{-13} was observed (Urban and Baeuerle, 1990). Presumably, gel purification removed impurities from the preparation which interfered with binding.

Comparative Analysis of NF-κB-binding Sites—We have listed 23 currently known binding sites for NF-κB that were also shown to be cis-acting elements. Using methylation interference analysis or related techniques, most NF-κB-binding sites were found to be decameric (listed in Baeuerle and Baltimore, 1991). This is compatible with NF-κB recognizing two pentameric half-sites by a p50 homodimer (Baeuerle and Baltimore, 1989). Some κB sites show, however, undecameric (IL-2 receptor, Böhnlein *et al.*, 1988) or even dodecameric[3] (lymphotoxin) patterns of methylation interference. This might be due to the fact that one NF-κB complex can recognize, within a DNA sequence of 11 or 12 base pairs, various decameric motifs which all fulfill consensus requirements. For such sites, we have listed all possible decameric motifs.

From a preliminary inspection it was apparent that there is always one half-site with three fully conserved guanosine residues while the other one is more degenerated. Binding sites are therefore arranged such that the half-site with the three subsequent G residues is on the left (Fig. 6). (In a few cases, we show κB motifs as they are present on the opposite strand or on both strands.) From the base frequency for a

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[3] Messer, G., Baeuerle, P. A., and Weiss, E. H., *Cytokine*, in press.

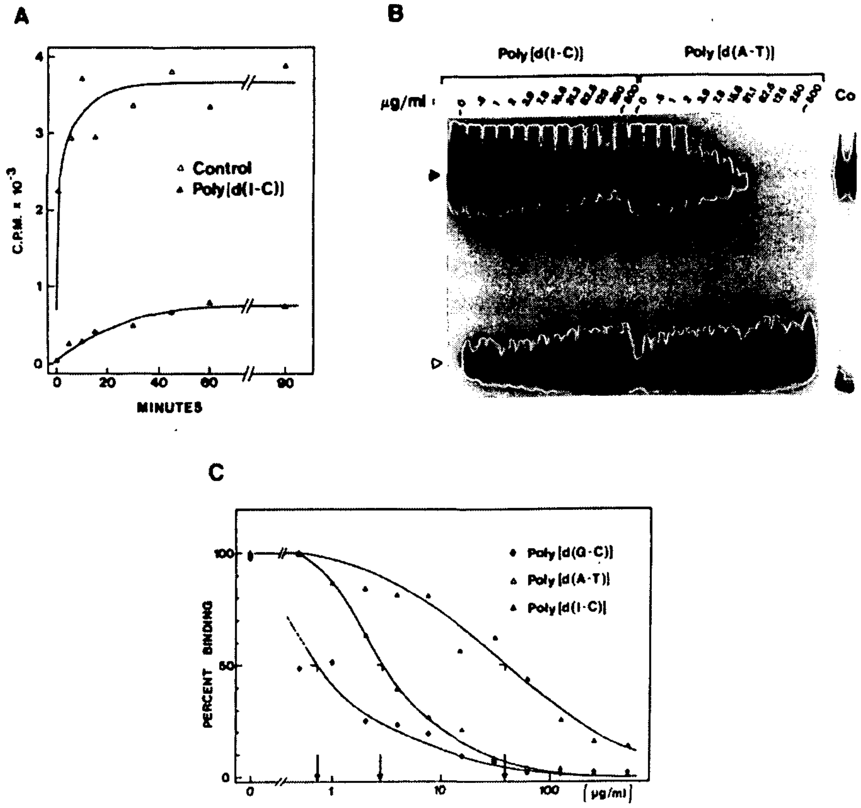


FIG. 4. **The effect of synthetic polynucleotides on specific DNA binding of NF-κB and on binding kinetics.** *Panel A*, binding kinetics. Purified NF-κB was reacted for various times in the absence (control) or presence of 0.1 mg/ml poly(dI-dC). Binding reactions were stopped by electrophoretic separation of samples on native gels. For the 0 min values, NF-κB was added and the sample immediately electrophoresed. From observing the migration of the bromphenol blue tracking dye, which comigrates with the free DNA probe, we assume that the protein-DNA complex was separated from free DNA within approximately 1 min from the start of the electrophoresis. *Panel B*, the effect of polynucleotides with alternating base sequence on the formation of protein-DNA complexes. Increasing amounts of poly(dI-dC) or poly(dA-dT) were added together with the labeled κB probe to purified NF-κB. In the *last lane* of the panel, a gel lane is shown in which purified NF-κB was assayed in the absence of poly(dI-dC) and with excess of DNA probe. Fluorograms of native gels are shown. The *filled arrowhead* indicates the position of the NF-κB-DNA complexes, the *open arrowhead* the position of free DNA. *Panel C*, dose-response curves for poly(dI-dC), poly(dG-dC), and poly(dA-dT). The radioactivity in NF-κB-DNA complexes is plotted as percent of control samples without polynucleotides. Fifty percent inhibitory concentrations are indicated by *arrows*.

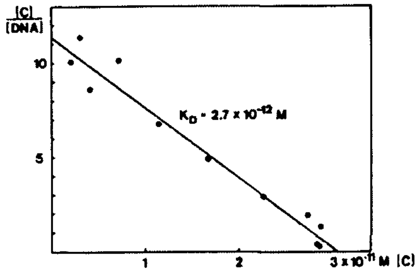
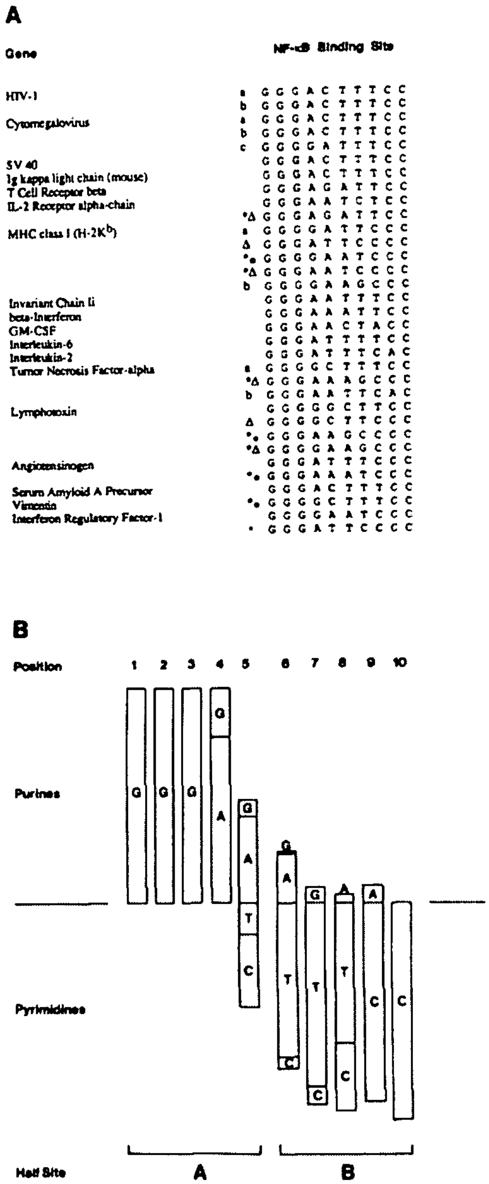


FIG. 5. **Scatchard analysis of NF-κB-DNA binding.** Purified NF-κB was titrated with various amounts of a radioactive κB probe with a specific activity of 60,000 Ci/mmol. Binding was performed as described under "Experimental Procedures" and samples analyzed by EMSA. Calculations of concentrations were based on the number of DNA-binding sites of NF-κB determined in the presence of a high excess of the κB probe.

given position of the decameric motifs, it is obvious that the two half-sites have different degrees of sequence conservation (Fig. 6*B*). The A half-site contains 4 fully conserved purine residues (positions 1–4) 3 of which are guanosines (1–3). In the B half-site only position 10 with a C residue is completely conserved. Position 9 which has in most cases a C can also have an A apparently without impairing the biological activity of the site (Hoyos *et al.*, 1989; Collart *et al.*, 1990). While there is always a G residue in position 3 of half-site A, the equivalent position 8 in half-site B can be variable. Also position 7 is more degenerate in contrast to the respective position 4 of half-site A because three different nucleotides can be tolerated in this position. The bases in the center of the motif (positions 5 and 6) can be variable with a preference for T residues in position 6. Although the half-sites show a high preference for either purines or pyrimidines, respectively (Fig. 6*B*), a dyad symmetry is restricted in the majority of sites to only the 2 outermost residues and to one or, at most, two internal bases (see Fig. 6*A*). A functional NF-κB site of ideal symmetry such as 5′-GGGACGTCCC-3′ or 5′-GGGAATTCCC-3′ has not been reported yet. Because the half-sites of the κB motif show different degrees of conservation they are likely to also differ in their interaction with the p50 monomers in the NF-κB complex and subsequently in their contribution to the affinity of protein-DNA interaction.

NF-κB Binds to Various Physiological κB Motifs with Dis-



Gene		NF-κB Binding Site
HIV-1	a	G G G A C T T T C C
	b	G G G A C T T T C C
Cytomegalovirus	a	G G G A C T T T C C
	b	G G G A C T T T C C
	c	G G G G A T T T C C
SV 40		G G G A C T T T C C
Ig kappa light chain (mouse)		G G G A C T T T C C
T Cell Receptor beta		G G G A G A T T C C
IL-2 Receptor alpha-chain		G G G A A T C T C C
	*Δ	G G G A G A T T C C
MHC class I (H-2K^b)	a	G G G G A T T C C C
	Δ	G G G A T T C C C C
	*a	G G G G A A T C C C
	*Δ	G G G A A T C C C C
	b	G G G G A A G C C C
Invariant Chain Ii		G G G A A T T T C C
beta-Interferon		G G G A A A T T C C
GM-CSF		G G G A A C T A C C
Interleukin-6		G G G A T T T T C C
Interleukin-2		G G G A T T T C A C
Tumor Necrosis Factor-alpha	a	G G G G C T T T C C
	*Δ	G G G A A A G C C C
	b	G G G A A T T C A C
Lymphotoxin		G G G G G C T T C C
	Δ	G G G G C T T C C C
	*a	G G G A A G C C C C
	*Δ	G G G G A A G C C C
Angiotensinogen		G G G A T T T C C C
	*a	G G G A A A T C C C
Serum Amyloid A Precursor		G G G A C T T T C C
Vimentin	*a	G G G G C T T T C C
Interferon Regulatory Factor-1		G G G G A A T C C C
	*	G G G A T T C C C C

FIG. 6. **Comparative analysis of known cis-acting NF-κB-binding sites.** *Panel A*, alignment of currently identified κB motifs in 5′-3′ orientation. κB motifs of genes that possess more than one site are labeled *a*, *b*, or *c*. *, κB sites as found on the antisense strand; Δ, κB sites obtained by shifting a decameric frame over sites with 11 or 12 base pairs. For most references see Lenardo and Baltimore, (1989) and Baeuerle and Baltimore (1991). Not yet reviewed therein are angiotensinogen (Ron *et al.*, 1990), vimentin (Lilienbaum *et al.*, 1990) and interferon regulatory factor-1.[4] *Panel B*, base composition of NF-κB binding motifs. Each *bar* represents one of the 10 sequence positions of the κB motif and the length of a *bar* corresponds to a total of 23 sites. For the nucleotide composition, those sites that allow binding of NF-κB in more than one way were divided by the number of possibilities.

tinguishable Affinity—Purified NF-κB was reacted with a labeled oligonucleotide encompassing sequences from the mouse κ light chain enhancer in the presence of 2.5- 25-, and 250-fold molar excesses of unlabeled double-stranded competitor oligonucleotides (Fig. 7). The oligonucleotides encompassed κB motif-containing sequences from the enhancers of

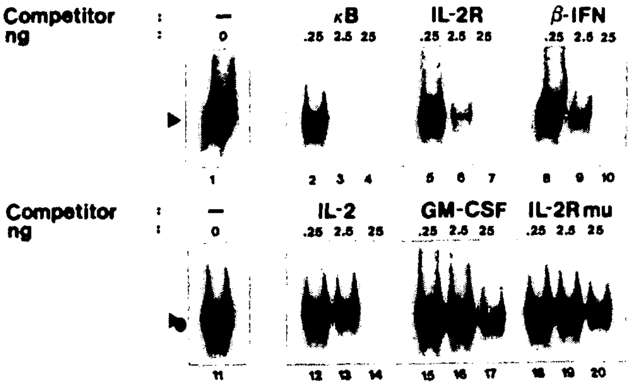


FIG. 7. **Competition analysis with various κB motifs.** Binding of purified NF-κB to a radioactively labeled κ light chain enhancer DNA probe (*κB*, 0.1 ng/reaction) was competed with 2.5-, 25-, and 250-fold molar excesses of unlabeled double-stranded oligonucleotides encompassing NF-κB-binding sites from other genes. *IL-2R*, interleukin-2 receptor α-chain enhancer; *β-IFN*, β-interferon enhancer; *mu*, mutant. Sections of fluorograms from native gels are shown. The *filled arrowhead* indicates the position of NF-κB-DNA complexes.

the mouse κ light chain gene (Sen and Baltimore, 1986) and human IL-2 receptor α-chain (Böhnlein *et al.*, 1988), β-interferon (Visvanathan and Goodbourn, 1989; Lenardo *et al.*, 1989), IL-2 (Hoyos *et al.*, 1989), and granulocyte macrophage-colony stimulating factor genes (Schreck and Baeuerle, 1990). There were significant differences between the various oligonucleotides with respect to their efficiency to inhibit binding of the radioactive DNA probe to NF-κB. The homologous competition with the κB oligonucleotide (κB motif: 5′-GGGACTTTCC-3′) was most effective (Fig. 7, *lanes 2–4*). The IL-2 receptor (5′-GGGAATCTCC-3′), β-interferon (5′-GGGAAATTCC-3′), and IL-2 (5′-GGGATTTCAC-3′) sites showed in that order decreasing competition efficiencies (Fig. 7, *lanes 5–14*) indicating that NF-κB had lower and distinguishable affinities for these κB motifs. The amount of complex formed in the presence of 2.5 ng of cold κB oligonucleotide (Fig. 7, *lane 2*) was similar to that obtained in the presence of 25 ng of IL-2 oligonucleotide (*lane 13*) suggesting that NF-κB binds with an approximately 10-fold lower affinity to the IL-2 site. A weak competition was observed with a 250-fold molar excess of the κB-related site 5′-GAGATTCCAC-3′ found in the granulocyte macrophage-colony stimulating factor gene (Schreck and Baeuerle, 1990) (Fig. 7, *lanes 15–17*). This site has only a weak, if any, cis-acting effect (Miyatake *et al.*, 1988). A mutated IL-2 receptor α-chain site (5′-GGGAATCTAA-3′) showed the lowest efficiency to compete (Fig. 7, *lanes 18–20*). Most likely, the adenosine residues in position 2 of the granulocyte macrophage-colony stimulating factor site and in positions 9 and 10 of the mutated IL-2 receptor α-chain site interfered with the ability of the oligonucleotides to compete binding because they replace residues which are highly conserved in κB motifs (see Fig. 6). An adenosine in position 9 is more tolerable as seen with the IL-2 site. A completely unrelated oligonucleotide showed no competition within the concentrations tested above (data not shown).

Using competition experiments, we have also tested whether purified NF-κB can interact with the two single-stranded oligonucleotides used to anneal the κ light chain enhancer oligonucleotide probe (data not shown). The single-stranded oligonucleotides were denatured by boiling prior to addition to the DNA-binding reactions. Only a 25,000-fold molar excess of upper and lower strands (approximately 2.5 μg of oligonucleotide) showed a significant inhibition of NF-κB binding to the double-stranded probe. The lower strand

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[4] R. Schreck, C. Winter, and P. A. Baeuerle, manuscript in preparation.

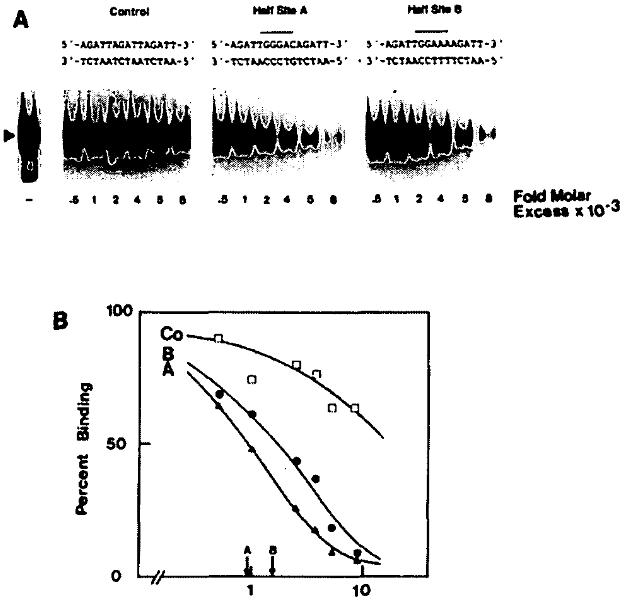

FIG. 8. **Competition analysis with half-sites of the κB motif.** *Panel A*, EMSA showing the competition efficiencies of three oligonucleotides. Three double-stranded 15-mers, shown on the top of the *panel*, were used which contain the pentameric half-sites of the κ enhancer motif in their center (indicated by a *bar*) flanked by the same sequence, AGATT. The control oligonucleotide contained three copies of the flanking sequence. Sections of fluorograms from native gels are shown and the concentrations of unlabeled competitior oligonucleotides indicated on the bottom. The *filled arrowhead* indicates the position of the NF-κB-DNA complexes. *Panel B*, quantitation of the competition analysis. *Open squares*, control; *filled circles*, half-site B; *filled triangles*, half-site A.

from the mutated IL-2 receptor α-chain site had almost no effect. We consistently observed that the upper strand (5'-GGGACTTTCC-3') competed more strongly than the lower strand (5'-GGAAAGTCCC-3') suggesting that nucleotides of the upper strand are more important for the protein-DNA interaction than those on the lower strand. On the assumption that the competition efficiency is proportional to the binding affinity, the dissociation constants of complexes of NF-κB with the single strands were approximately a thousand-fold lower than that for the respective double-strand, *i.e.* in the order of 10^{-9} M.

κB Half-sites as Competitors of NF-κB DNA Binding—To directly test the possibility that NF-κB has a distinguishable affinity for the two half-sites of the κB motif 5'-GGGACTTTCC-3', we used in a titration experiment double-stranded 15-mer oligonucleotides encompassing isolated half-sites of the motif within the flanking sequence 5'-AGATT-3' (Fig. 8*A*). The unspecific control oligonucleotide used consisted of three copies of the flanking sequence. The oligonucleotide encompassing half-site A could compete for binding of NF-κB to the labeled probe approximately two times more efficiently than that containing half site B (Fig. 8*B*) suggesting that the more highly conserved half-site A is recognized with higher affinity by p50 than half-site B. The competition profiles were reproducible and reflected a specific binding of NF-κB to the isolated half-sites because the control oligonucleotide showed a significantly weaker competition. The competition efficiency of the half-sites was in the same order of magnitude as that obtained with complete single-stranded sites.

DISCUSSION

The Influence of Monovalent Cations on the Activity of NF-κB—At low concentrations of the NF-κB protein, the formation of a NF-κB·DNA complex was enhanced by increasing the concentration of NaCl or KCl presumably reflecting a higher binding affinity of NF-κB to DNA in the presence of monovalent cations. An explanation for this effect might come from the capability of DNA-binding proteins to displace upon their binding monovalent cations from the DNA backbone (Record *et al.*, 1976). Due to a gain in entropy through releasing ions from DNA, a net increase in binding energy is obtained at elevated salt concentrations. Above 100 mM, a chaotropic effect of the salts might prevail leading again to reduced binding.

The Role of Zn^{2+} in DNA Binding of NF-κB—We demonstrate here the requirement of Zn^{2+} for the DNA binding activity of NF-κB. This finding could indicate that NF-κB recognizes DNA using zinc finger motifs (for reviews see Klug and Rhodes, 1987; Evans and Hollenberg, 1988). It is interesting to note that a recently cloned DNA-binding protein which specifically recognizes κB motifs contains functional Zn-finger motifs (Fan and Maniatis, 1990; Baldwin *et al.*, 1990). This protein with a size of almost 300 kDa has, however, various features that distinguish it from NF-κB. It is also conceivable that zinc ions in addition to or instead of forming zinc finger structures help to cross-link the DNA binding p50 dimer. A role for zinc ions in dimerization was recently proposed for *tat*, the transactivator protein of human immunodeficiency virus type 1 (Frankel *et al.*, 1988). Only the determination of the primary structure of the p50 subunit of NF-κB and a subsequent deletion and mutation analysis will allow to approach this question.

High Affinity DNA Binding by NF-κB—The affinity of NF-κB for the site 5'-GGGACTTTCC-3' with a K_D in the order of 10^{-12} to 10^{-13} M is extremely high. This might provide one explanation why the protein-DNA complex of NF-κB was insensitive toward the intercalating effect of chloroquine. We noticed, however, that the preincubation of DNA with chloroquine had almost no effect on binding of NF-κB to the DNA (Fig. 1*A*). It is therefore possible that chloroquine either did not intercalate in the κB site, or, that NF-κB was able to displace DNA-intercalated chloroquine. We consider it less likely that NF-κB could form a high affinity complex with DNA containing an intercalated substance.

The high binding constant of NF-κB might be important for two characteristics of the transcription factor. One is its low abundance within cells of less than 1,000 DNA-binding sites/cell (Lenardo *et al.*, 1989; Baeuerle and Baltimore, 1989) which requires a much higher affinity to recognize target sites than needed by a more abundant DNA-binding protein such as NF1 (Meisterernst *et al.*, 1988). This protein binds to its respective cognate sequences with an affinity constant in the order of 10^{-10} M. The other is the biological function of NF-κB to rapidly induce gene expression upon extracellular stimuli that signal distress and pathogen invasion (Baeuerle and Baltimore, 1991). Rapid and persistent induction of gene expression requires a fast on-rate and high affinity of protein binding to cis-acting elements as reported in this study. The affinity by which NF-κB binds to a site might be important to regulate by the average occupation time of a cis-acting element the rate of transcriptional initiation of a particular gene. In this line, it would be interesting to determine the relationship between K_D value and rate of transcription initiation for the various κB motifs.

Differential Half-site Recognition by NF-κB—We propose that the two p50 molecules in the NF-κB complex can interact differentially with the half-sites of most decameric κB motifs. This idea is supported by the following observations. (i) The half-sites of known cis-acting κB motifs exhibit different degrees of sequence conservation. The pentameric half-site A is completely conserved in the first three positions whereas half-site B only in one position. (ii) Upper and lower strands of the tested κB motif showed different efficiencies of binding competition. The upper strand competed better presumably because it contains the 3 fully conserved G residues found in all known κB motifs. As suggested by the strong competition of binding by poly(dG-dC), G residues appear to be most important for protein-DNA interaction. (iii) Single pentameric half-sites of the κB-binding site can specifically compete for binding of NF-κB to a decameric site whereby the more conserved half-site A showed a 2-fold stronger competition than the less conserved half-site B. While one p50 molecule in the NF-κB complex interacts strongly and with similar affinity with the A half-sites of κB motifs, the interaction of the second p50 molecule with the less conserved B half-sites appears to determine the fine affinity of a given site.

Necessary for a high affinity interaction of NF-κB with a DNA motif appear to be the nucleotides found in positions 1, 2, 3, 4, and 10 because they are most highly conserved. The bases in positions 5, 6, 7, 8, and 9 allow more variation suggesting that they might determine the subtle differences in affinity found with the various κB sites (Fig. 2). We consider it unlikely that NF-κB contains a DNA-binding heterodimer because the p50 subunits appear to be indistinguishable (Baeuerle and Baltimore, 1989).

There is an increasing number of DNA-binding proteins that can also specifically recognize κB-like sequence motifs including KBF1 (Yano *et al.*, 1987), H2TF1 (Baldwin and Sharp, 1988), MBP-1/PRDII-BF1 (Baldwin *et al.*, 1990; Fan and Maniatis, 1990) and EBP-1 (Wu *et al.*, 1988; Clark *et al.*, 1989). Many of the data presented in this characterization of the DNA binding activity and specificity of NF-κB will be helpful to distinguish these related factors from NF-κB and to examine cloned proteins that exhibit sequence-specific binding to κB motifs.

Acknowledgments—We are grateful to Gerald Messer and Drs. Lienhart Schmitz and Haralabos Zorbas for stimulating discussions, Dr. Jeremy Wells for helpful comments on the manuscript, Andrea Oswald for synthesizing oligonucleotides, Ulrike Wagner and Claudia Winter for technical assistance, and Prof. Dr. E.-L-Winnacker for his continuous support.

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Nucleic Acids Research, 1993, Vol. 21, No. 8 1959–1964

The transcriptional enhancer element, κB, regulates promoter activity of the human neurotropic virus, JCV, in cells derived from the CNS

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Received November 6, 1992; Revised and Accepted March 16, 1993

ABSTRACT

Studies on the regulation of the human neurotropic virus (JCV) promoter, have been focused primarily on the 98 bp tandem repeat sequence which confers glial-specificity to viral gene expression. We demonstrate that a distinct regulatory element outside of the 98 bp region, which spans a stretch of 10 nucleotides (nt) (5'-GGGAATTTCC-3') increases transcriptional activity of JCV late (JCV_L) and early (JCV_E) promoters in glial cells. Sequence analysis of this motif reveals extensive homology to the κB sequence of HIV-1 (5'-GGGACTTTCC-3'). A DNA fragment corresponding to the 10 nt sequence of JCV exhibits transcriptional activity when placed upstream of the test promoter in glial cells. The induction mediated by this regulatory motif is moderately enhanced in response to phorbol 12-myristate 13-acetate (PMA) in glial cells. Band-shift and UV-crosslinking experiments suggest that glial cells constitutively produce proteins that specifically interact with the JCV κB, but not the HIV-1 κB motif. Treatment of cells with PMA results in formation of new complexes that are sensitive to the κB sequences derived from the JCV and HIV-1 genomes. These results suggest that the κB sequence located in the JCV genome may play a role in transcriptional regulation of JCV gene expression by interacting with inducible and uninducible nuclear proteins from glial cells.

INTRODUCTION

The JC virus (JCV), a member of the genus polyomavirus, is a human papovavirus etiologically linked to a fatal demyelinating disease of the central nervous system (CNS) termed Progressive Multifocal Leukoencephalopathy (PML) (1–12). This virus exhibits close sequence homology to the well-characterized human papovaviruses, simian virus (SV40), and the BK virus (BKV) (13, 14). JCV and BKV share approximately 80% homology between their viral early proteins (T- and t-antigens) and up to 80% homology between the late viral capsid proteins (VP1, VP2, and VP3). JCV and SV40 share approximately 70% homology in these regions. However, unlike BKV and SV40, JCV has a narrow tissue tropism that has hampered the study of this virus in culture. JCV replicates efficiently in primary human fetal glial cell cultures rich in spongioblasts, the presumed precursor of oligodendrocytes (15, 16). Several human cells (embryonic kidney, amnion, and urine-derived epithelium) have been shown to poorly support viral replication (17–19).

The greatest degree of divergence between JCV, BKV, and SV40 is in the viral control region which in JCV contains the origin of DNA replication, promoters for early and late gene transcription, and tandem 98-base pair (bp) direct repeats. Several lines of evidence indicate that the restricted tissue specificity of JCV is determined by this region of the virus. Analyses of viral gene expression by transient transfection studies and in transgenic animals have revealed that the tissue tropism of JCV resides, at least in part, in the glial-specific activation of the viral early and late promoters (20–23). Evidently, the 98-bp repeats of the JCV regulatory sequence function as tissue-specific enhancers for these promoters.

Our laboratory has initiated a detailed examination of the cis- and trans-regulatory factors that restrict JCV gene expression to glial cells. Studies by us (24–26) and others (27, 28) have suggested that the JCV control region contains multiple protein binding domains that interact with distinct factors present in glial and non-glial cells (24–28). Figure 1 illustrates the structural organization of the JCV regulatory region and the positions of DNA-binding proteins and cis-acting elements. Use of hybrid promoter constructs in transient transfection studies have revealed that at least two of these domains positively and negatively contribute to the specific transcription of a heterologous promoter in glial and non-glial cells, respectively. Thus, it is likely that transcription of the viral early promoter is under positive and negative regulation in permissive and non-permissive cells. In support of these findings, results from cell fusion experiments have indicated that expression of the viral early promoter was extinguished in the nuclei of glial cells when these cells were

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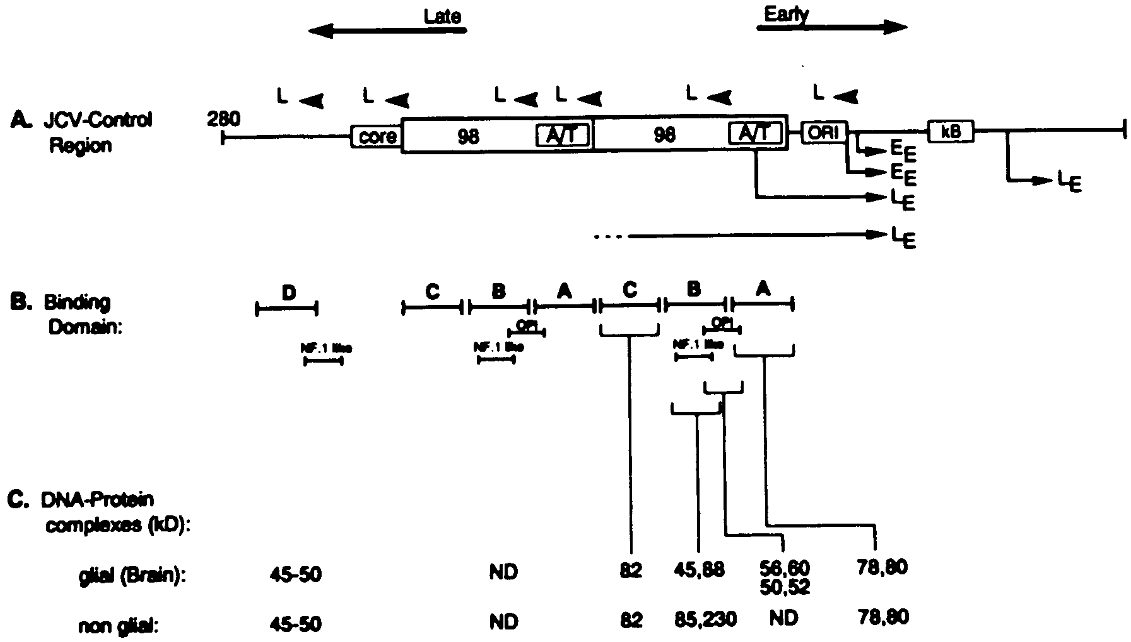


Figure 1. Schematic diagram of the transcription control region of JCV. **A.** Control elements including the origin of viral DNA replication (ori), the AT-rich (TATA box) sequence, the core region representing an octameric motif of SV40, (GTGCAAAG), and the κB box (GGGAATTTCC) are positioned between the early (encoding large T- and small T-antigens) and the late (encoding viral structural proteins $VP_{1,\ 2,\ \text{and}\ 3}$) translation initiation sites. The arrows indicate start sites of viral early RNAs before DNA replication (E_E) and after DNA replication (L_E) (41, 42). The positions of the late RNA initiation sites are shown by arrowheads (43). **B.** Locations of protein binding regions and cis-acting regulatory elements (24–28) as examined by various DNA-binding techniques and transient transfection assay. **C.** The relative molecular mass of the complexes formed in glial and non-glial nuclear extracts as examined by UV-crosslinking techniques (24). ND, not determined.

fused with fibroblasts (29). This observation suggests that a negative transdominant factor in non-glial cells directly or indirectly suppresses expression of the JCV early promoter.

Earlier studies by Major, et al (30) have identified a putative κB sequence on the early side of the viral origin (Figure 1). κB is a potent transcription enhancer which was first identified in the kappa-light chain of immunoglobulin gene, and later in many inducible cellular and viral transcription units (31). This enhancer, which is composed of 10 nucleotides (nt) (GGGACTTTCC), binds to a large family of proteins called Nuclear Factor-Kappa B (NFκB). NFκB is a complex of two proteins of 50 and 65 kD that is thought to bind DNA either as a heterodimer of P50/P65 or as a homodimer of P50/P50 (31). These proteins pre-exist in the cytoplasm of most cells in an inactive form complexed to an inhibitor, termed IκB (31). Stimulation by a number of agents such as phorbol-12 myristate-13 acetate (PMA), lipopolysaccharide (LPS), or tumor necrosis factor-α (TNFα), results in the dissociation of the IκB-NFκB complex, presumably by a protein kinase C-dependent phosphorylation of IκB (31). The unbound NFκB is, in turn, transported to the nucleus where it binds to its target sequence and exerts its activity.

In the present report, we have investigated the biological effects of the 10 nt κB sequence homologous to the κB transcription element of HIV-1 on the expression of the JCV early and late promoters in human glioblastoma cells. We now demonstrate that this κB sequence induces overall activities of the JCV late and to a lesser extent, early promoters in glial cells. Results from binding studies revealed the association of multiple inducible and non-inducible nuclear proteins from glial cells that differentially bind to JCV-κB and a classical κB sequence derived from the HIV-1 promoter.

EXPERIMENTAL PROCEDURES

Plasmid DNA constructs

The plasmids, pJCV$_L$ CAT and pJCV$_E$ CAT contain the 286 bp Hind III-Pvu II fragment representing the overlapping regulatory sequences of the JCV late and early genes, cloned upstream of the chloramphenicol acetyl transferase (CAT) gene (32). pJCV$_E$κB-CAT and pJCV$_L$κB_CAT plasmids were constructed by placing a 388 bp polymerase chain reaction (PCR) product representing the JCV regulatory region upstream of the CAT gene in the pBL-CAT$_3$ vector (33). The primers used in the PCR were 5′-CCTCCCTATTCAGCACTTT-3′, and 5′-GGCTCG-CAAAACATGTTCCC-3′. The template used to amplify DNA differs from the published sequence for MAD-1 strain of JCV in that it contains a small deletion in the second 98 bp repeat (nt 110–132). Such disparate nt alterations are apparently a common feature of JCV (K. Dorries, personal communication) and do not affect cell-specific transcription of viral promoters in glial cells.

Deoxyoligonucleotides of JCV-κB (5-GGGAATTTCC-3′) were synthesized using phosphoramidite chemistry and purified by gel electrophoresis. Complementary oligonucleotides were treated with polynucleotide kinase, annealed, and cloned into the pBL-CAT$_2$ expression vector at the Sal 1 site upstream of the HSV-TK promoter (33). The presence of the oligonucleotide insert was verified by DNA sequencing (34).

Transient transfection and CAT assay

Human glioblastoma cells, U-87MG (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). Cells were seeded at

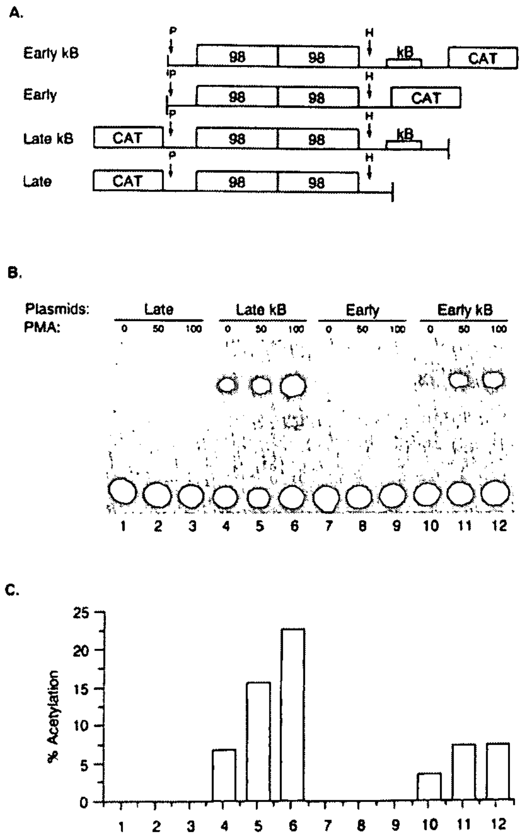


Figure 2. Transcriptional activity of JCV-κB in glial cells. A. JCV_E and JCV_L constructs contain a 288 bp Hind III-Pvu II DNA fragment of the JCV regulatory sequence in early and late orientations, respectively, placed in front of the CAT gene (32). JCV_E κB and JCV_L κB constructs contain a 388 bp DNA sequence from JCV that includes a 286 bp stretch from the Hind III site to the Pvu II site plus 102 nt overlapping the early side of the replication origin. The 388 bp DNA fragment was generated by polymerase chain reaction (PCR) with appropriate primers in which Bam H1 restriction sites were incorporated as cloning sites. B. Approximately 5×10^5 human glioblastoma cells (U-87MG) were transfected with 4 μg of plasmid DNA by Ca-phosphate precipitation technique (35). After 4 hours, cells were washed with PBS and refed with fresh medium containing 50 or 100 ng/ml PMA. Cell extracts were prepared 36–48 h post transfection and CAT enzymatic activity was determined (37). C. The percent conversion of chloramphenicol to its acetylated forms was determined by liquid scintillation counting of substrate and acetylated forms.

5×10^5 cells per 60 mm plate and 20 h later 4 μg of test plasmid DNA was introduced into the cells by the Ca-phosphate precipitation procedure (35). The final amount of DNA in each transfection mixture was brought to 15 μg with pUC DNA.

At 4 h post-transfection, cells were subjected to glycerol shock (36). Cells were washed several times with PBS and incubated with fresh medium in the absence or presence of PMA at concentrations of 50 or 100 ng/ml. All experiments were performed independently at least three times. Cells were harvested at 36–48 h post-transfection and assayed for CAT activity as described (37). Percent conversion of [^{14}C]chloramphenicol to its acetylated form was determined by liquid scintillation counting of the spots cut from the TLC plates.

Nuclear extract preparation and band-shift assay

Nuclear extracts were prepared from U-87MG cells as described previously (38). For band-shift experiments, the oligonucleotide probe containing the JCV-κB sequence (5′-GGGAATTTCC-3′) was end-labeled with [γ^{32}P]ATP, annealed, and purified on a 9% native acrylamide gel. Approximately 15,000 cpm of probe was incubated with approximately 10 μg of nuclear extract protein in the presence of 25 mM Hepes (pH 7.9), 40 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.4 mg/ml BSA, 3 mM GTP, 0.3 mg/ml poly[dI-dC], and 10% glycerol (v/v) in a final volume of 30 μl at 4°C for 30 min. Competition experiments were carried out in the presence of unlabeled JCV-κB, HIV-1-κB, and HIV-1-κB^{mut} DNA fragments. In this case, the extract was preincubated with the competitor DNA for 10 min prior to the addition of the labeled probe. Complexes were electrophoresed on 6% polyacrylamide gel (prerun at 150 v for 2 h) at 150 v for 4–5 hours with continuous circulation of running buffer at 4°C. Gels were fixed in 10% acetic acid, 10% methanol, dried and exposed to Kodak X-AR film at −70°C with intensifying screen.

UV-crosslinking

Labeled oligonucleotides (JCV-κB) were mixed with extracts under conditions as described in the band-shift assay. Reaction mixtures were exposed to UV light (302 nm) for 30 min at 4°C and resolved by SDS-PAGE. The gel was dried and autoradiographed as described to determine the relative molecular mass of the complexes.

RESULTS AND DISCUSSION

Activation of JCV promoters by κB sequence in glial cells

To gain insight into the glial-specific expression of the JCV genome, and to examine the possible role of the sequence located downstream from the enhancer/promoter, we carried out a series of transient transfection experiments utililizing plasmids containing the reporter chloramphenicol acetyl transferase (CAT) gene fused to various regions of the JCV regulatory sequence. Figure 2A schematizes the structure of the recombinants which were used in these studies. The constructs were introduced into human glioblastoma cells (U-87MG) by the Ca-phosphate precipitation method (35) and CAT enzymatic activities were measured 48 h after transfection. The results shown in Figure 2B indicate that the sequence positioned downstream from the 98-bp repeat increases transcription of viral late and early promoters by 10- and 5-fold, respectively, in glial cells (compare lanes 1 and 7 with 4 and 10, respectively). Inspection of the sequences located upstream of the 98-bp repeats has revealed a stretch of 10 nt (5′-GGGAATTTCC-3′) with significant homology to the κB enhancer of HIV-1 (5′-GGGACTTTCC-3′). It is now well-established that the κB sequence binds to a family of transcription factors which are induced with a variety of agents, such as phorbol esters, and enhances transcriptional activity of the linked promoter (31). To examine the inducibility of the JCV-κB sequence, cells were maintained in medium containing phorbol-12-myristate B-acetate (PMA) after transfection, and transcriptional activities of the JCV early and late promoters were examined by CAT assay. As shown in Figure 2B, treatment of cells with PMA revealed approximately 2- to 3-fold elevation in the activity of the κB-containing constructs (lane 5, 6, and 11, 12). In parallel studies, the κB sequences show less than two-fold enhancement of the JCV early and late promoter activity in Hela cells (data not shown). Treatment of these cells with PMA had no effect on viral early and late promoter activities. These studies suggest that the upstream sequence of JCV encompassing

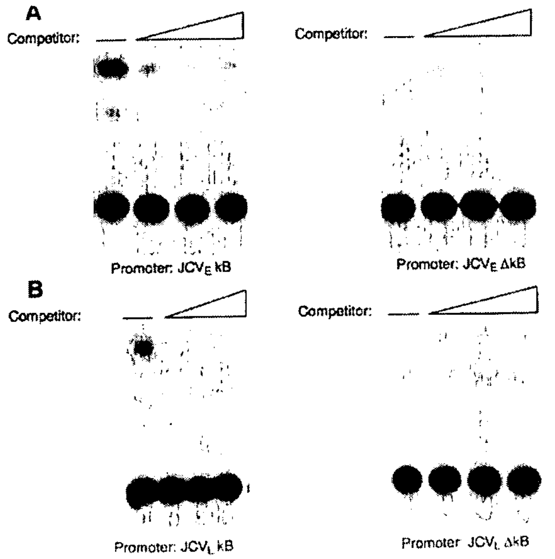


Figure 3. Co-transfection competition analysis of the JCV promoter in U-87MG glial cells. The JCV late and JCV κB reporter constructs containing various lengths of the JCV early and late regulatory sequences, respectively (as detailed in Figure 2A) were transfected alone or together with increasing amounts (250-, 500-, and 1,000-fold molar excess) of competitor oligonucleotide fragment containing the JCV κB sequence (5′-GGGAATTTCC-3′). Cellls were harvested 48 h post-transfection and CAT assay was determined (37).

the κB motif has the capacity to augment the basal activity of viral early and late promoters in U-87MG glial cells. Evidently, this sequence responds positively to NFκB inducers such as PMA. However, the modest induction of these promoters upon PMA treatment suggests the involvement of a trans-regulatory factor(s) that is constitutively active in these cells.

Next, to determine more specifically whether the κB sequence plays a role in the activity of the intact JCV promoters, in vivo competition experiments were carried out. To this end, the synthetic oligonucleotide derived form JCV κB was co-transfected with the reporter constructs containing JCV early and late regulatory sequence and the level of CAT activity was determined. In the presence of 250-, 500-, and 1,000-fold molar excess of κB sequence, JCV early and late promoter activities were substantially reduced (Figure 3) suggesting that the κB sequence is important for enhanced transcriptional activity of JCV promoters and that there are other regions of JCV that are necessary to maintain viral gene transcription at high levels in glial cells under similar conditions. The κB sequence exhibits no significant effect on transcriptional activity of viral promoters with no κB sequence (Figure 3). These data imply that the κB sequence is operative in the context of the viral promoter, and increases both early and late promoters.

Transcription of the JCV promoters in glial cells

To verify that the increase in CAT activity of the κB containing plasmids was due to increased levels of CAT transcripts, the level of CAT RNAs was measured by S1 nuclease protection assay (39). In this study, a 458 nt single-stranded S1 probe containing a sequence complementary to the CAT coding sequence was uniformly labeled and hybridized to total RNAs derived from cells transfected with various JCV constructs (as shown in Figure 4). S1 nuclease digestion by CAT RNA is expected to result in a protected fragment of 256 nt. Results shown in Figure 4 illustrate the protection of an appropriately cleaved 256 nt DNA fragment corresponding to protection by RNA transcribed only in cells transfected with the κB plasmids. These results demonstrate that the κB-sequence stimulates transcription of JCV early and late promoters in glial cells.

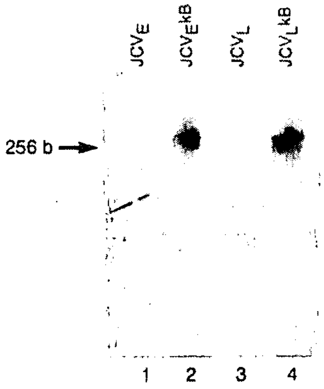


Figure 4. S1 nuclease analysis of RNA from cells transfected with JCV$_E$ and JCV$_L$ promoters. At 48 h post transfection, total cellular RNA was isolated from cells transfected with constructs containing JCV$_E$ and JCV$_L$ and hybridized to a single-stranded uniformly labeled CAT DNA probe. S1 digestion was carried out by the method described previously and protected DNA fragments were purified and analyzed by electrophoresis in a denaturing sequencing gel as described previously (39). The arrow indicates the position of the 256 nt protected fragment.

Activation of a heterologous promoter in glial cells by JCV-κB sequence

To further examine the transcriptional activity of the JCV-κB sequence, we synthesized a double-stranded oligonucleotide harboring the JCV-κB sequence, GGGAATTTCC, and fused it to the 5′-end of a test promoter derived from Herpes simplex virus thymidine kinase (TK) (33) in both early and late orientations, separately. This promoter contains several transcriptional elements, i.e. TATA box, GC-rich sequences and a CAAT box (40). Transfection experiments were carried out in the presence or absence of PMA, and CAT enzyme production was measured after 48 h. As shown in Figure 5, basal CAT activity, due to expression of the TK promoter, was significantly increased in glial cells when the JCV-κB sequence was linked to the test TK promoter. The level of enhancement was significantly higher when the κB sequence was placed in the late orientation. Treatment of the cells with PMA showed only a two-fold increase in the CAT activity of constructs containing κB in the late orientation and had an insignificant effect on the construct with κB in the early orientation. A low level of induction ($<50\%$) of TK promoter activity was detected in Hela cells transfected with the hybrid promoter harboring JCV-κB in the late orientation (data not shown). These results suggest that the κB sequence of JCV is operative and has the capacity to increase transcriptional activity of a fused heterologous promoter in glial cells.

Formation of specific DNA protein complexes by the JCV-κB sequence

In an attempt to identify the cellular factors present in glial cells that interact with the JCV-κB sequence,. band-shift experiments

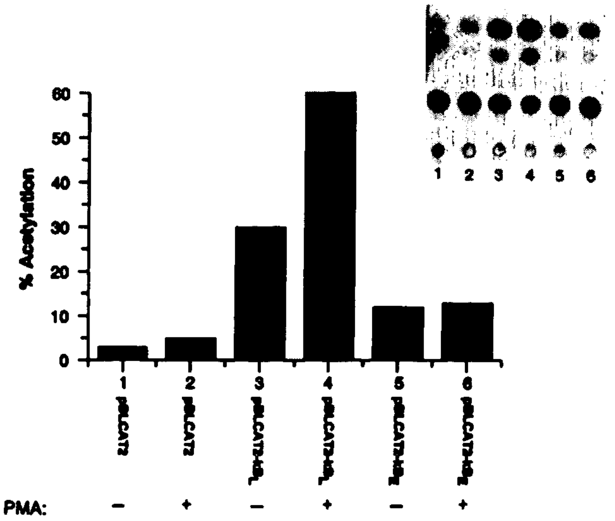
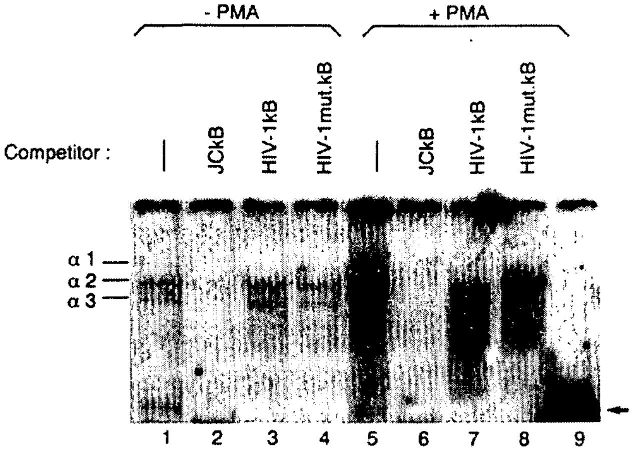


Figure 5. Functional analysis of the JCV-κB sequence. Synthetic double-stranded DNA (oligo-JC κB) homologous to the JCV-κB sequence was placed in either orientation, in front of the Herpes simplex virus thymidine kinase gene promoter which contains a TATA box, GC-rich sequence and CAAT box (40). The control and each of the recombinant constructs were introduced into U-87MG glial cells in the presence or absence of PMA by the Ca-phosphate precipitation procedure (35) and enzymatic activity of the CAT protein expressed from these plasmids was determined (37). The percent of chloramphenicol converted to its acetylated forms was determined as described in Figure 2 legend.



Competitor Oligonucleotides

JCkB	5'-GGGAATTTCC-3'
HIV-1kB	5'-GGGACTTTCC-3'
HIV-1mut.kB	5'-CTCACTTTCC-3'

Figure 6. Protein-binding analysis of the JCV-κB sequence. Nuclear extracts were prepared from U-87MG glial cells that were treated with PMA as previously described (31). Approximately 15,000 cpm of [^{32}P]-end-labeled double-stranded oligonucleotide fragment were incubated in 30 μl of nuclear extract reaction mixture containing 10–12 μg of protein as described earlier (36). The resulting complexes were resolved on a 6% native polyacrylamide gel. In competition studies, unlabeled DNA fragments (>100-fold molar excess) were added to each reaction prior to incubation with the probe. Lane 9 represents probe alone.

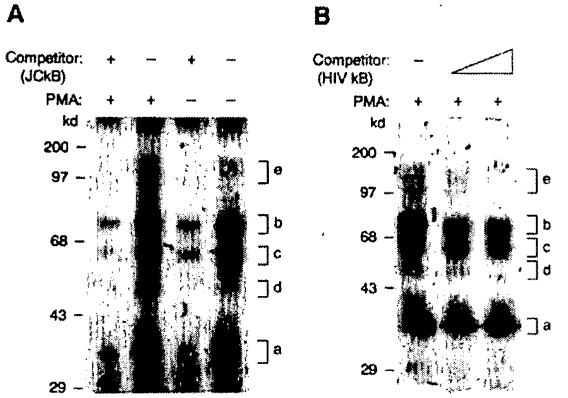


Figure 7. Identification of the κB binding protein by UV-crosslinking. UV-crosslinking and competition assays were carried out as described previously (36). Briefly, the double-stranded JCV κB oligonucleotide was uniformly labeled and UV-crosslinked to nuclear protein derived from glial cells PMA-treated and untreated in the presence or absence of unlabeled JCV κB (A) and HIV-1 κB (B) sequences.

were performed. In this study, double-stranded JCV-κB oligonucleotide was end-labeled by T4-polynucleotide kinase in the presence of [γ^{32}P]ATP and incubated with nuclear extracts derived from U-87MG cells in the presence or absence of PMA. The extract from untreated cells produced two closely migrating complexes, α_2 and α_3. Addition of excess unlabeled JCV-κB oligonucleotides (>100-fold) abolished formation of these complexes, whereas under similar conditions, the κB sequence from the HIV-1 promoter containing only one nucleotide mismatch revealed no significant effect on the intensity of the α_2 and α_3 bands (Figure 6, lanes 1–3). Expectedly, the mutant HIV-1-κB oligonucleotide (GGGACTTTCC changed to CTCACTTTCC), which is functionally inactive (31), was unable to block the formation of the α_3 complex (Figure 6, lane 4). The PMA-treated glial extract showed an elevated level of α_2 complex (or a complex that co-migrates with α_2) and the appearance of a new complex α_1 (Figure 6, lane 5). All three forms (α_1, α_2, and α_3) were sensitive to excess amounts of unlabeled homologous competitor sequence. Preincubation of the extract with HIV-1-κB competitor reduced the intensity of the α_2 complex to the level seen in untreated extract, and abolished formation of the α_1 complex. The addition of unlabeled HIV-1-κB mutant to the extract showed no significant effect on the intensity of the α_1, α_2, and α_3 complexes (Figure 6, lane 8). These results suggest that, in addition to the two inducible complexes which recognize both JCV and HIV-1-κB sequences, glial cells may contain nuclear proteins non-responsive to PMA that preferentially recognize the JCV-κB sequence.

Identification of JCV-κB binding proteins by UV-crosslinking

To further characterize the nuclear proteins that participate in binding to the JCV-κB sequence, UV-crosslinking experiments were employed. Binding reactions were carried out according to the band-shift procedure as described above using 20 μg of nuclear protein from U-87MG and PMA-treated U-87MG cells. Complexes were cross-linked for 30 min at 4°C using long wave UV light (302 nm). The resulting complexes were directly analyzed by SDS-PAGE. As shown in Figure 7, three major complexes, a, b, and c, migrating as broad bands in the ranges of 35, >68, and 56 kD, respectively, were reproducibly detected in the untreated extract. In the presence of PMA, additional complexes, d and e, with relative molecular masses of 48 and 87–102 kD, respectively, were observed. Addition of 100-fold excess of unlabeled JCV-κB DNA significantly reduced the intensity of the bands corresponding to all five complexes (Figure 7A, lanes 2 and 4). Preincubation of the extracts with unlabeled HIV-1-κB DNA reduced the intensity of the inducible 48 and 87–102 kD complexes, but showed virtually no effect on the formation of 35, >68, and 56 kD complexes (Figure 7B).

At the present time, the role of these complexes in the activation of the JCV promoter in glial cells is unclear. However, the profile of transcription activation of the viral promoters containing the JCV-κB sequence upon PMA treatment, and the formation of specific NFκB complexes in PMA-treated extract, suggest that the participant proteins in the 35, >68, and 56 kD complexes may be involved in the overall activation of the JCV early and late promoters. Similarly, the proteins that form the 48 and 87–102 kD complexes may mediate transcription enhancement of JCV gene expression upon PMA induction.

In summary, experiments described here demonstrate that the sequences homologous to the NFκB transcriptional element present within the JCV genome have the capacity to stimulate expression of the bidirectional viral promoter in glial cells. This regulatory element, which differs in only one nt with the classic κB sequence of HIV-1, binds to constitutive and inducible DNA binding proteins present in glial cells. Experiments are in progress to examine the importance of this regulatory motif in the physiology of the JCV life cycle in cells treated with a variety of agents, such as LPS, cAMP, cytokines, tumor necrosis factors α and β (TNFα and TNFβ), transforming growth factor β (TGFβ), and interleukin 1. Results from these studies should enhance our current knowledge of the mechanisms that trigger replication and/or expression of latent JCV in immunocompromised individuals.

ACKNOWLEDGEMENTS

We are grateful to Dr S.Amini and members of the Molecular Neurovirology Section of the Jefferson Institute of Molecular Medicine for sharing their reagents and for their support and helpful suggestions during the course of this study. We thank Ms. Jennifer Gordon for critical reading of this manuscript and Ms. C.Schriver for preparing this manuscript. This work was supported by Grants AI 28272 and CA 47996 awarded by the National Institutes of Health to K.Khalili, and by Grant 2324-A-25 awarded by the National Multiple Sclerosis Society to K. Khalili.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 7, Issue of February 18, pp. 4705–4708, 1994
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Printed in U.S.A.

Communication

Role of Transcription Factor NF-κB/Rel in Induction of Nitric Oxide Synthase*

(Received for publication, November 5, 1993, and in revised form, December 10, 1993)

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The promoter of the murine gene encoding inducible nitric oxide synthase (iNOS) contains an NF-κB site beginning 55 base pairs upstream of the TATA box, designated NF-κBd. Reporter constructs containing truncated promoter regions, when transfected into macrophages, revealed that NF-κBd is necessary to confer inducibility by bacterial lipopolysaccharide (LPS). Oligonucleotide probes containing NF-κBd plus the downstream 9 or 47 base pairs bound proteins that rapidly appeared in the nuclei of LPS-treated macrophages. The nuclear proteins bound to both probes in an NF-κBd-dependent manner, but binding was resistant to cycloheximide only for the shorter probe. The proteins binding both probes reacted with antibodies against p50 and c-rel but not RelB; those binding the shorter probe also reacted with anti-RelA (p65). Pyrrolidine dithiocarbamate, which acts as a specific inhibitor of NF-κB, blocked both the activation of the NF-κBd-binding proteins and the production of NO in LPS-treated macrophages. Thus, activation of NF-κB/Rel is critical in the induction of iNOS by LPS. However, additional, newly synthesized proteins contribute to the NF-κBd-dependent transcription factor complex on the iNOS promoter in LPS-treated mouse macrophages.

Nitric oxide (NO) is reported to participate in the physiology or pathophysiology of every organ system (1). Of the three mammalian gene products known to act as NO synthases, the high output isoform, termed iNOS[1] (2), is the most widely expressed in various cell types (1) following its transcriptional induction (2). Among the most important stimuli for induction of iNOS is bacterial endotoxic lipopolysaccharide (LPS) (3, 4). Induction of iNOS by LPS contributes to the pathogenesis of septic shock (5). Moreover, induction of iNOS by other stimuli leads to organ destruction in some inflammatory (6) and autoimmune diseases (7). At the same time, iNOS plays a key role in host defense against infectious agents of almost every known class (8), including viruses (9, 10). Thus, it is of interest to understand how expression of iNOS is regulated.

The recently cloned promoter of the murine gene coding for iNOS (11) contains at least 22 elements homologous to consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines and LPS. Among these are two putative NF-κB binding sites, one upstream (GGGATTTTCC, nucleotides −971 to −962, designated NF-κBu) and one downstream (GGGACTCTCC, nt −85 to −76, designated NF-κBd). The consensus sequence for NF-κB binding sites, GGGRNNYYCC (12), allows for 128 versions of the motif, of which about 20 have been reported, the most common being GGGACTTTCC (12). NF-κBu is identical to the NF-κB site in the interleukin-6 promoter (12). In contrast, NF-κBd has been found only in iNOS. We reported earlier (11) that chloramphenicol acetyltransferase (CAT) constructs containing fragments of the iNOS promoter region from nt −721 to +161 or from −478 to +161, both of which contained several consensus sequences besides NF-κBd, conferred inducibility of the iNOS promoter by pathophysiologically relevant concentrations of LPS (11). The present report uses additional constructs to demonstrate that NF-κBd is necessary for LPS inducibility of the iNOS promoter, characterizes and compares the nuclear factors that bind to the NF-κBd element in a restricted and in a more extended context, and presents evidence that NF-κB/Rel plays a critical role in the induction of the endogenous iNOS gene by LPS.

MATERIALS AND METHODS

Cell Culture—The macrophage cell line RAW 264.7 (American Type Culture Collection) was grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin.

Reagents—Recombinant murine IFN-γ was a gift of Genentech (South San Francisco, CA). LPS, cycloheximide, and pyrrolidine dithiocarbamate (PDTC) were from Sigma. Poly(dI-dC)·poly(dI-dC) was from Pharmacia LKB Biotechnology Inc. and isotopes from Amersham Corp. Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) supplied affinity-purified rabbit IgGs against synthetic peptides derived from the following members of the NF-κB/Rel family: p50, RelA (p65), c-rel (residues 152–176; designated c-rel(N)), c-rel (residues 498–517; designated c-rel(C)), and RelB.

Plasmids—The plasmid p1*iNOS-CAT* (11) was digested with *Sac*I, and the linear product was treated with nuclease Bal-31 for 5 min. The resulting fragments were self-ligated and transformed into *Escherichia coli* strain HB101. One clone contained plasmid p8.1*iNOS-CAT*, which had a deletion of 407 bp (−492 to −86) from p1*iNOS-CAT*, generating a *Sma*I site adjacent to the 5′ end of NF-κBd. The promoter region upstream of this *Sma*I site was removed to form p8.11*iNOS-CAT*. To construct p8.13*iNOS-CAT*, the region −75 to +161 was amplified by polymerase chain reaction and the product cloned into p*CAT-Basic*. Transfection and polymerase chain reaction assays were as described (11).

Oligonucleotides and Probes—Single-stranded oligonucleotides (Oligos Etc., Inc., Guilford, CT) were annealed by polymerase chain reaction to form oligomers A, M, and B below. Uppercase sequences are from the promoter. The NF-κBd site is underlined; mutations are italicized. To prepare probe A or B, oligomer A or B was filled in by the Klenow fragment of DNA polymerase I with [α-^{32}P]dGTP or [α-^{32}P]dCTP and the three other nonradiolabeled dNTPs.

−85
5′gaagct TGGGGACTCTCCCTTTG
ACCCCTGAGAGGGAAACCCTT

OLIGOMER A

5′TG*CTC*ACTCTCCCTTTGGGA
*GAG*TGAGAGGGAAACCCTTGTC

OLIGOMER M

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* This work was supported by National Institutes of Health Grant AI/CA 34543. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: iNOS, inducible nitric oxide synthase whose activity is independent of [Ca^{2+}] above the level found in resting cells; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; IFN-γ, interferon-gamma; LPS, endotoxic bacterial lipopolysaccharide; NF-κB, nuclear factor for immunoglobulin κ chain in B cells; PDTC, pyrrolidine dithiocarbamate; bp, base pair(s).

5′caTGGGGACTCTCCCTTTGGGAACAGTTATGCAAAATAGCTCTGCAGAGCCTGGAGGGG
CCCTGAGAGGGAAACCCTTGTCAATACGTTTTATCGAGACGTCTCGGACCTCCCCAgct

OLIGOMER B

Electrophoretic Mobility Shift Assay (EMSA)—Binding reactions (15 µl total) were performed by incubating 5 µg of nuclear extract prepared as described (13) with reaction buffer (20 mM HEPES pH 7.9, 1 mM EDTA, 60 mM KCl, 12% glycerol, 4 µg of poly(dI-dC)·poly(dI-dC)) in the presence or absence of competitor or antibody for 10 min, followed by a 20–25-min incubation at room temperature with probe (~20,000 cpm). Products were electrophoresed at 30 mA for 3 h on 4.8% polyacrylamide gels in high ionic strength buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH ~8.5) (14) and dried gels analyzed by autoradiography.

RESULTS

NF-κBd Is Necessary for LPS Inducibility of iNOS Promoter—In the reporter construct p8.11*iNOS-CAT* (-85 to +161), all regions upstream from NF-κBd have been deleted. Upon transfection into RAW 264.7 cells, p8.11*iNOS-CAT* displayed promoter activity that was induced by LPS in a concentration-dependent manner (Fig. 1), as with the full-length promoter/enhancer (11). When the 10-bp NF-κBd site was removed (construct p8.13*iNOS-CAT*; -75 to +161), LPS scarcely induced promoter activity (Fig. 1).

LPS Induces Binding of p50, c-Rel, and p65 (RelA) to NF-κBd Site—We next characterized the nuclear proteins in LPS-treated macrophages that bound to sequences from the iNOS promoter in an NF-κBd-dependent manner. Electrophoretic mobility shift assays were performed with probe A, an oligonucleotide consisting of the NF-κBd element and 9 more bp downstream. To explore the effects of context, we also used probe B, consisting of probe A plus the next 38 bp downstream. Treatment of RAW 264.7 cells with LPS led to the appearance in their nuclei of protein(s) that bound to both probes (Fig. 2*A*, complex I on probe A and complex X on probe B). Other bands (complex II on probe A and unlabeled bands on probe B) were detected in nuclei from cells with or without LPS treatment. Formation of complexes I, II, and X was blocked specifically by an excess of nonradiolabeled oligomer A, but not by oligomer M, in which the first 3 base pairs of the NF-κBd sequence are mutated. A 42-bp oligonucleotide containing one tandem repeat of GGGGACTTTTCC (catalog no. 3382SA, Life Technologies, Inc.) also competed, while an unrelated oligonucleotide derived from the coding region of iNOS did not (not shown). Complex I appeared within 0.5 h and diminished within 4 h following the addition of LPS, while the more slowly migrating complex X appeared later and persisted for at least 6 h (Fig. 2*B*).

In other studies with activated macrophages using rather short NF-κB probes (Ref. 15 and citations therein), the EMSA pattern closely resembled that in Fig. 2*A* with probe A. In those studies, it was speculated that complex I contained a heterodimer of p50 and p65 subunits, while complex II was thought to contain a p50 homodimer. In the present work, as shown in Fig. 3, both complexes I and II were supershifted dramatically when the nuclear extract was preincubated with

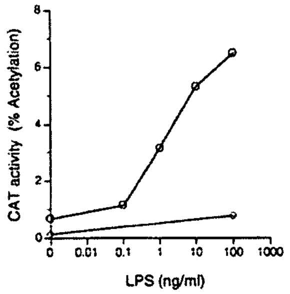

FIG. 1. **Inducibility of iNOS promoter in reporter constructs containing or lacking NF-κBd.** CAT activity was determined in RAW 264.7 cells transfected with p8.11*iNOS-CAT* (○) or p8.13*iNOS-CAT* (◇) after treatment with the indicated concentrations of LPS. Results were similar in two additional experiments.

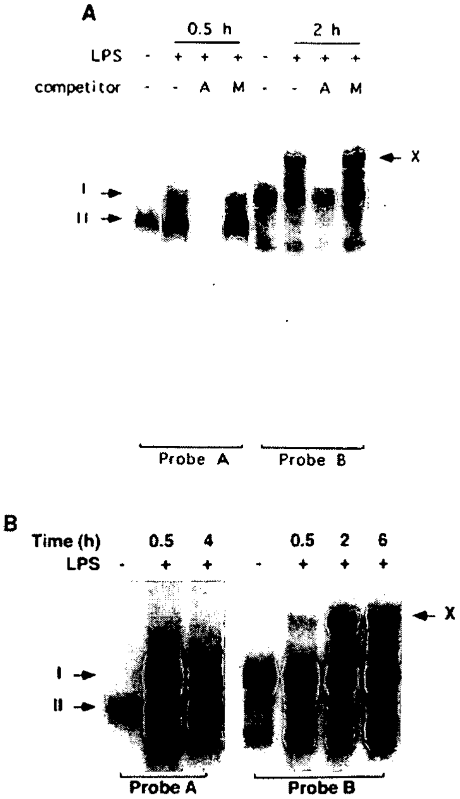

FIG. 2. **NF-κBd-dependent binding of nuclear proteins in macrophage cell line RAW 264.7.** The cells were treated with LPS (100 ng/ml) for the indicated times before preparing nuclear extracts for analysis by EMSA with probe A or B as shown. *A*, specificity. Binding was carried out in the absence or presence of a molar excess of oligomer A or oligomer M (500-fold with probe A and 250-fold with probe B). *B*, time course.

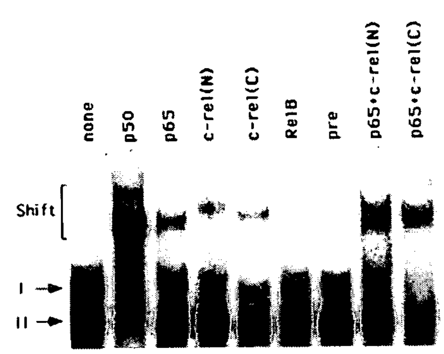

FIG. 3. **Identification of NF-κBd-binding proteins in nuclei of RAW 264.7 cells treated with LPS (100 ng/ml).** Nuclear extracts were incubated in the absence (*none*) or presence of 1 µg of preimmune serum (*pre*) or affinity-purified IgGs against p50, RelA (p65), the amino terminus of c-rel (*c-rel(N)*), the carboxyl terminus of c-rel (*c-rel(C)*), RelB, or combinations as indicated, followed by a binding reaction with probe A.

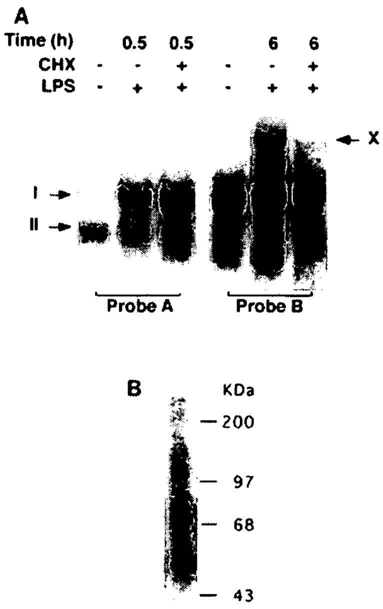

FIG. 4. **Effects of cycloheximide and UV cross-linking analysis of nuclear NF-κBd-binding proteins in RAW 264.7 cells.** *A*, cells were treated with LPS (100 ng/ml) in the absence or presence of cycloheximide (*CHX*, 1 μg/ml) for the indicated times. Nuclear extracts were then prepared and subjected to EMSA using either probe A or B. *B*, cells were treated with LPS (100 ng/ml) for 6 h and nuclei then collected. In a scaled-up EMSA, 50 μg of nuclear extract and 10^6 cpm of bromodeoxyuridine-containing probe B were reacted in a total volume of 75 μl. The EMSA gel was irradiated at 254 nm from 5 cm for 30 min at 4 °C. The band corresponding to complex X was excised, treated with DNase I, and analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The migration of M_r standards is indicated.

IgG against NF-κB p50 before binding to probe A. Less extensive supershift was observed with IgGs against c-rel(C), c-Rel(N), or RelA (p65), but a combination of IgGs against c-rel(C) and RelA (p65) supershifted complex I completely. Thus, complex I appears to include mixed heterodimers of p50/c-rel and p50/RelA (p65), while complex II appears to consist predominantly of p50. In contrast, no supershift was observed with IgG against RelB or with preimmune IgG (Fig. 3). When probe B was used, supershift of complex X was observed with IgGs against p50 or c-rel but not RelA (p65) or RelB (not shown).

Formation of Cycloheximide-sensitive DNA-Protein Complex on iNOS Promoter—NF-κB/Rel proteins exist preformed in the cytoplasm of macrophages until their post-translational activation and nuclear translocation (12, 16). Consistent with this, the ability of LPS to induce binding of NF-κB/Rel proteins to probe A was unaffected by treating the macrophages with the protein synthesis inhibitor cycloheximide. In contrast, cycloheximide markedly reduced the formation of complex X on probe B (Fig. 4*A*). UV cross-linking analysis revealed that complex X contains polypeptides migrating with apparent molecular mass of 50, 65–75, and 115 kDa (Fig. 4*B*).

NF-κB Is Involved in Induction of iNOS Gene—PDTC, an antioxidant that acts as a specific inhibitor of NF-κB activation in LPS-treated macrophages (16), blocked both the ability of LPS to induce nuclear binding activity for NF-κBd and the ability of macrophages to produce nitrite in response to LPS alone or LPS plus IFN-γ (Fig. 5).

DISCUSSION

We conclude that the downstream NF-κB site in the iNOS promoter, a unique sequence termed NF-κBd, is necessary to confer inducibility by LPS in mouse macrophages. A nuclear protein complex that binds specifically to NF-κBd after treatment of macrophages with LPS contains the heterodimer p50/c-rel. Transcriptional regulation by c-rel has been reported only rarely (17).

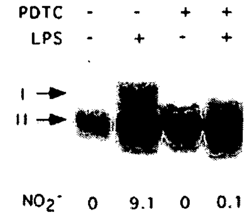

FIG. 5. **Effect of PDTC on protein binding to NF-κBd and cellular production of nitrite.** RAW 264.7 cells were preincubated with or without PDTC (60 μM, 1 h) and then treated with or without LPS (100 ng/ml) for 0.5 h (EMSA) or 8 h (nitrite accumulation). Their nuclear extracts were subjected to EMSA with probe A and their conditioned media analyzed for nitrite (NO_2^-, nmol/10^6 cells) by the Griess assay (2). When IFN-γ (10 ng/ml) was included along with LPS, nitrite accumulation increased to 24.3 nmol; pretreatment with PDTC reduced this to 3.4 nmol. In all cases, cell viability exceeded 80% by trypan blue exclusion.

Since PDTC, a relatively specific inhibitor of the activation of NF-κB in macrophages (16), blocked both the production of nitrite and the binding of NF-κB/Rel to NF-κBd, it appears that NF-κB/Rel is involved in the induction not just of iNOS-derived reporter constructs but of the iNOS gene itself in LPS-treated macrophages. In other studies, PDTC blocked nitrite/nitrate production by a rat macrophage cell line treated with LPS and IFN-γ (18). The related antioxidant diethyldithiocarbamate blocked NO release and expression of iNOS mRNA in LPS-stimulated mouse macrophages (19). However, neither study tested the effect of the antioxidants on binding of NF-κB/Rel proteins to the iNOS promoter.

Assignment of a critical role to NF-κB/Rel in the induction of iNOS at first seemed paradoxical. Cycloheximide inhibits the induction of mRNA for iNOS in mouse macrophages (20, 21), so that a protein(s) newly synthesized in response to LPS must be necessary for induction of iNOS in this setting. However, cycloheximide did not inhibit the binding of NF-κB/Rel to NF-κBd. The paradox was resolved by the use of a more extended segment of the iNOS promoter. This revealed that the NF-κBd-dependent binding of complex X was cycloheximide-sensitive (Fig. 4*A*). Complex X includes multiple polypeptides (Fig. 4*B*), some of which may be newly synthesized after LPS induction and then interact with NF-κB/Rel proteins to form a cycloheximide-sensitive complex. Indeed, there are at least five other transcription control proteins with which members of the NF-κB/Rel family are known to interact, namely Sp1 (22), Fos/Jun (23), NF-IL6 (24), HMG I(Y) (25), and TATA-binding protein (26). Analysis of the cycloheximide-sensitive determinant(s) of complex X should lead to better understanding of the molecular mechanisms for regulation of iNOS and possibly other NF-κB/Rel-regulated genes.

These results raise the possibility that iNOS may be inducible by some of the numerous stimuli known to activate NF-κB besides LPS (12, 27). These include oxidative stress (18, 28) and viruses (12). The latter possibility is of particular interest in view of the ability of NO to inhibit viral replication (9, 10).

Acknowledgments—We thank Dr. Hsiou-Chi Liou for helpful advice, Richard Whisnant for technical help in the experiments illustrated in Fig. 1, and Aihao Ding for critique of the manuscript.

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Regulation of the NF-κB/rel transcription factor and IκB inhibitor system

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The interplay between proteins of the NF-κB/rel and IκB families is a tightly regulated process that ensures appropriate responses to specific environmental and developmental signals. Various mechanisms are utilized in regulating NF-κB/rel and IκB activities, some unique to this transcription factor system. All of these regulatory strategies converge towards one purpose, namely the controlled nuclear translocation of activated NF-κB/rel protein complexes. The variety of rel-related and ankyrin repeat containing subunits makes regulation of this system both rich and complicated.

Current Opinion in Cell Biology 1993, 5:477–487

Introduction

NF-κB was first identified as a DNA-binding activity specific for the κB motif in the immunoglobulin κ light chain enhancer in B lymphocytes. Decameric κB-like recognition sites have since been found in many genes involved in immune function and acute phase responses. The cloning of the genes encoding the two subunits of NF-κB, p50 and p65, revealed a family of NF-κB/rel proteins that participates in various transcriptionally controlled processes, such as cytokine responsiveness, lymphoid differentiation and embryonic axis determination in insects [1,2].

The NF-κB/rel proteins are subject to multiple regulatory influences. A major component of this regulation is the control of their intracellular localization, with inactive protein maintained in the cytoplasm and transcriptionally active protein transported to the nucleus. Cytoplasmic retention of inactive forms of NF-κB/rel is achieved through interaction with a group of inhibitor molecules, the IκBs. Two members of the NF-κB/rel family are regulated by proteolytic processing from precursors. In addition, the family is subject to more common regulatory strategies, such as post-translational modification, particularly by phosphorylation.

In this review, we focus on various means by which NF-κB/rel activity is regulated in response to extracellular stimuli or during stages of differentiation, especially B-cell development. The intrinsic differences between various NF-κB/rel complexes determine target gene specificities and transcription activities. As NF-κB/rel activity is tightly maintained in the cytoplasm in most cells, deregulation and/or imbalance among different members of NF-κB/rel and IκB families can potentially cause disorders of the immune systems, including leukemia and autoimmune diseases.

Nomenclature of NF-κB/rel and IκB gene family

The cloning of the p50 and p65 subunits of the NF-κB heterodimer showed them to be members of a family of proteins, the NF-κB/rel family, all of which are involved in gene regulation [3,4]. They share a Rel-homology (RH) region of approximately 300 amino acids over which they have about 54% identity. The known protein members of this family are NF-κB p50 and p65, the p105 precursor of p50, p52 and its p100 precursor (also called lyt-10), c-Rel, RelB and Dorsal (Table 1). The RH domain is responsible for many of the key activities of the NF-κB/rel proteins, including DNA binding site recognition, dimerization, nuclear localization and interaction with IκBs.

Table 1. Nomenclature of NF-κB/rel and IκB families.

Common name	Protein	Gene
NF-κB/rel:		
NF-κB	p50	*NFKB-1*
	p65	*relA*
	p105	*NFKB-1*
Lyt-10 (p100)	p100	*NFKB-2*
	p52	*NFKB-2*
c-rel	c-Rel	*c-rel*
relB	RelB	*relB*
dorsal	Dorsal	*dorsal*
IκB:		
IκB-α	IκBα	*MAD-3*
IκB-β	IκBβ	(not yet cloned)
IκB-γand CTR	IκBγ	*NFKB-1*
Bcl-3	Bcl-3	*bcl-3*

Abbreviations

CTR—carboxyl-terminal region; **EMSA**—electrophoretic mobility shift assay; **LPS**—lipopolysaccharides; **PHA**—phytohemagglutinin; **PKA**—protein kinase A; **PKC**—protein kinase C; **PMA**—phorbol myristate acetate; **RH**—rel homology; **TNF**—tumor necrosis factor.

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The existence of this protein family was first appreciated through sequence comparisons of Dorsal and Rel; the linkage to NF-κB was made when the gene for the p105 precursor of p50 was cloned. A number of homologues were then identified. The names for the genes involved have recently been standardized by general agreement among workers in the field and are given in Table 1. The p105 precursor is an especially interesting molecule because it contains both activator and inhibitor functions: the amino-terminal half encodes p50, a subunit of the NF-κB transcription-activating complex, while the carboxyl-terminal region contains ankyrin-like repeats, which are commonly found in the IκB family of proteins (see below). The p100 precursor was cloned as the putative oncogene, *lyt*-10, from a chromosomal translocation site in a B-cell lymphoma [5]. It has a similar structure to p105 and was also identified as an immediate-early activation gene of human peripheral blood T cells [6]. The other NF-κB/rel proteins are not cleaved from precursors but are translated into their final form. NF-κB p65 contains an acidic amino acid rich carboxyl terminus that mediates transcription activation. The proto-oncogene, c-*rel*, was identified through its oncogenic derivative, v-*rel*, found in an avian retrovirus, REV-T. The v-*rel* derivative induces fatal B-cell lymphomas in infected chickens. *relB* was isolated as a serum-induced gene from a mouse fibroblast library [7]. It is most related to c-*rel*, but contains a putative leucine zipper domain in a long amino terminal segment as well as a carboxyl-terminal transcriptional activation domain. The *Drosophila* morphogen, *dorsal*, is involved in the control of dorsal–ventral axis formation in the fly [8].

The IκB family proteins are defined by their ability to bind to one or more of the NF-κB/rel proteins. Members include IκB-α, IκB-β, IκB-γ [or the carboxyl-terminal region (CTR) of p105], the proto-oncogene *bcl*-3, and the *Drosophila* gene *cactus*. The IκB proteins so far identified contain between five and seven repeats of 33 amino acid 'ankyrin motif' involved in protein–protein interaction; the gene for IκB-β has yet to be cloned. IκB-α is the homologue of avian pp40 protein that co-immunoprecipitates with v-Rel in REV-T transformed lymphomas [9]. IκB-α is encoded by the mouse cDNA, *MAD*-3, first isolated from a macrophage library as an immediate-early response gene [10•]. The carboxyl-terminal region of p105 contains IκB-like activity and, at least in mouse B cells, is also encoded independently of the precursor by a separate mRNA [11,12]. The proto-oncogene, *bcl*-3, was identified at a 14:19 translocation breakpoint in the IgH-α region of a subset of adult chronic lymphocytic leukemias of B-cell lineage [13]. The *Drosophila* gene, *cactus*, was genetically defined as a negative regulator of *dorsal*. It was cloned by both by a P element trap method and by low-stringency screening with an ankyrin repeat probe [14•,15•].

The ankyrin repeats in IκB family proteins are necessary for interaction with the RH domains of the NF-κB/rel proteins, but the exact mode of binding is unclear. There is evidence that the interaction between IκB and NF-κB/rel proteins requires a functional dimerization ability in the RH domain, suggesting that IκB may associate with a dimeric NF-κB/rel complex. The nuclear localization signal in the RH domain is necessary for the interaction and is masked by IκB in the complex [16••,17••]. This explains, at least in part, the ability of the IκB molecules to keep the NF-κB subunits in the cytoplasm. There is specificity in the interaction as IκB-α exclusively inhibits dimers containing p65 and c-Rel, whereas IκB-γ (or CTR) and Bcl-3 preferentially interact with p50 and/or p52 [12,18].

Regulating NF-κB/rel and IκB activities

Nuclear translocation is necessary for transcription activation by NF-κB/rel proteins and is the convergence point for the regulatory mechanisms utilized by NF-κB/rel family. In most cases, nuclear translocation correlates with the biological function of NF-κB/rel proteins. For example, selective nuclear translocation of Dorsal protein is the key to the development of dorsal–ventral axis in the early embryos of *Drosophila* [8]. In the case of c-*rel* and its oncogenic partner v-*rel*, abnormal subcellular localization seems to play a part in the tumorigenesis caused by v-*rel* in REV-T transformed B-cell lymphoma and fibroblasts [19]. Treatment of cells with specific stimuli that phosphorylate and dissociate NF-κB/rel and IκB complexes, also results in nuclear translocation of active NF-κB/rel complexes. Because all mammalian cells tested have NF-κB/rel proteins, usually in a cytoplasmic reserve, the regulation of the family is particularly complex. The NF-κB/rel family is regulated by transcription, phosphorylation and probably proteolysis.

The NF-κB/rel proteins respond to many environmental stimuli [1], although the exact mode of induction of active nuclear NF-κB/rel complexes is unclear. For B lineage cells, lipopolysaccharides (LPS), phorbol myristate acetate (PMA), cAMP or cross-linking surface IgM can induce NF-κB/rel activity. For T cells, almost any stimuli capable of activation will elevate NF-κB/rel activity. These include PMA, tumor necrosis factor (TNF)-α, IL-1, IL-2, phytohemagglutinin (PHA), antigen engagement, and cross-linking surface CD2, CD3, CD28 and T-cell receptor. Infection of T cells by HIV-1, HSV and HTLV-1 also upregulates NF-κB/rel activity. In monocyte/macrophages, PMA, TNF-α, lymphotoxin and PHA and cross-linking CD2 are good inducers of NF-κB/rel activity. TNF and IL-1 are good activators for NF-κB/rel in fibroblasts and hepatomas.

NF-κB/rel activity is also regulated during B-cell differentiation. In pre-B cells, NF-κB/rel protein is kept at an inactive state although it can be activated by stimulating cells with PMA or LPS. By contrast, mature B cells constitutively express NF-κB/rel activity in the nucleus [20]. Constitutive NFκB/rel may be required to initiate Ig κ gene expression in B cells although in at least some plasmacytomas it is dispensable. The constitutive NF-κB/rel activity is thus under differentiation control in the B-cell lineage and there is also data to suggest that, as macrophages mature, it becomes constitutively active [21,22].

Given the complexity of this large family of transcription factors, the apparent NF-κB/rel activity observed in various systems may be composed of different dimers of the family even though migration rates may be indistinguishable when analyzed by the electrophoretic mobility shift assay (EMSA). Furthermore, each individual member of the family may have a different tissue distribution or may respond differently to a given stimulus. It is therefore worthwhile to discuss the possible ways by which individual members may be regulated (Table 2).

Table 2. Mechanisms of NF-κB/rel and IκB regulation.

- Phosphorylation and signal transduction pathways:
  - PKA, PKC, haem-regulated eIF-2 kinase
  - *Toll*→*tube*→*pelle*
  - TNF-α and IL-1β (ceramide)
  - Oxygen radicals
- Processing of the precursors:
  - p105 into p50
  - p100 into p52
- Tissue-specific and developmental expression:
  - p100 and p105 are constitutively transcribed but inducible
  - *c-rel* is specific to lymphoid tissues
  - p65 is ubiquitously expressed
- Combinatorial association and target gene specificity:
  - NF-κB has broad binding specificity and transcriptional activity
  - p52/p65 only activates certain κB sites
  - $(p50)_2$ and $(P52)_2$ selectively activate certain κB sites
  - c-Rel is an activator for IL-2R, but a repressor for IL-6
  - RelB forms heterodimers with p50 or p52 only
- Interaction with non-NF-κB/rel proteins:
  - IκBs
  - Bcl-3
  - NF-IL6
  - HTLV tax protein
  - HMG I(Y)
- Differential splicing:
  - IκB-γ
  - p65-delta

Phosphorylation and signal transduction pathways

Direct biochemical evidence indicates that phosphorylation of IκB can abrogate its binding to NF-κB and thereby control the appearance of active NF-κB/rel. This was demonstrated using purified IκB, which combined with NF-κB *in vitro* and prevented its binding to DNA as measured using EMSA [23]. Treatment of IκB with protein kinase A (PKA), protein kinase C (PKC) or the haem-regulated eIF-2 kinase can inactivate IκB as measured in this assay. This observation was further supported by the studies that purified IκB-α and IκB-β proteins are inactivated by phosphorylation with PKA and PKC [24]. Furthermore, IκB-β lost its inhibitory activity upon phosphatase treatment. Therefore, the activation of NF-κB may rely on both phosphorylation and dephosphorylation of IκB. Although the sites of phosphorylation have yet to be mapped, it is perhaps significant that IκB-α and IκB-γ contain a putative PKC site in their sixth ankyrin repeat. While no good PKA consensus sites have been found by sequence analysis, *in vitro* phosphorylation studies have demonstrated that IκB-α is a good substrate for PKA (HC Liou, D Baltimore, unpublished data). Thus PKA may phosphorylate a site different from the consensus PKA recognition sequence.

NF-κB/rel proteins are also likely to be targets of protein kinases based on their sequence analysis, which reveals several potential sites for protein kinase action. For example, at the carboxyl-terminal ends of all RH domains in NF-κB/rel proteins, there is a conserved consensus PKA recognition site 25 amino acids amino-terminal to the nuclear localization signal (NLS). Mutation of this PKA site in c-Rel and v-Rel proteins abolishes the transformation activity of v-Rel and relocalizes c-Rel from the cytosol to the nucleus [25,26]. Further studies, however, demonstrated that these loss-of-function mutants of v-Rel or c-Rel also fail to bind DNA because of a destruction of the dimerization domain, making it unclear whether the loss of function is due to the absence of phosphorylation or a conformational change of the protein [26].

In *Drosophila*, PKA or PKA-related kinases appear to regulate the establishment of dorsal–ventral polarity by the *Drosophila* morphogen, Dorsal. Dorsal protein is uniformly distributed throughout the cytoplasm of early embryos. Approximately 90 min after fertilization, Dorsal is selectively translocated to the nucleus in ventral, but not dorsal, regions [8]. Genetic approaches have identified 11 genes that control Dorsal activity. These genes are termed *dorsal* group genes because mutations of these genes keep Dorsal in the cytoplasm and make the embryo more 'dorsalized'. One of the dorsal group genes, *Toll*, encodes a surface receptor protein that contains a cytoplasmic tail with structural similarity to the mammalian IL-1 receptor. Both Toll and PKA can enhance the nuclear localization and transcription activity of Dorsal. This occurs through action at the potential PKA site because single point mutations at the PKA site abolish the responsiveness of Dorsal to induction by Toll or PKA [27••]. The structural similarity between the cytoplasmic tails of Toll and IL-1 receptor suggests that IL-1 may utilize PKA or a PKA-related kinase to regulate NF-κB/rel activity.

Among the 11 dorsal group genes, *tube* and *pelle* are genetically down-stream of *Toll* and upstream of *dorsal*. Interestingly, the recently cloned *pelle* cDNA contains a candidate kinase catalytic domain, which is similar to that found in the raf/mos family of protein kinases (S Wasserman, personal communication). The *tube* cDNA shows no obvious homology to any known genes [28]. It will be interesting to know if the *pelle* product is the kinase that catalyzes phosphorylation of the putative PKA site in Dorsal, and how the Tube and Pelle proteins modulate Dorsal–Cactus interaction.

Our knowledge about the signaling pathways for modulating NF-κB/rel and IκB through cytokine action and other agents is still rudimentary. However, some information about the second mediators or signaling molecules that are involved in NF-κB/rel regulation is just emerging. TNF-α and IL-1β are potent inflammatory cytokines and good NF-κB inducers in many cell types. Both TNF-α and IL-1β can apparently exert regulation on NF-κB through release of ceramide, which is generated by sphingomyelin breakdown catalyzed by sphingomyelinase [29•,30•]. Sphingomyelinase activity is controlled by 1,2-diacylglycerol, which is produced by a TNF-α responsive phosphatidylcholine–phospholipase C. Ceramide-activated protein kinase may belong to the family serine/threonine category of protein kinases, including MAP-2, EGF receptor threonine kinase, glycogen synthase kinase-3 and cdc2-related kinases [30•].

Active oxygen radicals may also play an important role in the activation of NF-κB activity. The strongest evidence for such a pathway is that antioxidants and metal chelators selectively block the activation of NF-κB by PMA, LPS, IL-1, TNF and possibly other stimuli [31•,32]. Could oxygen radicals or reactive oxygen intermediates be the direct regulators of NF-κB/rel proteins? There is an apparently zinc-coordinated structure in the RH domain involving several conserved cystines and histidines. Its integrity seems to be important because any deletions or point mutations involving these residues destroy the DNA-binding and dimerization activities of the proteins (H-C Liou, D Baltimore, unpublished data). It is therefore possible that oxygen radicals could oxidize key sulphydryl groups or that chelators could remove the zinc, but this seems an unlikely mode of regulation. Alternatively, the active oxygen radicals may act indirectly.

Processing of the precursors

The precursors of p50 and p52, the p105 and p100 products of the *NFKB1* and *NFKB2* loci, are bipartite molecules. While the amino termini include the transcription factor moieties, the carboxyl termini have ankyrin repeats, suggesting that they could be integral regulators. Consistent with this idea is the observation that if the p105 CTR is expressed separately, it acts as a p50-binding IκB [12]. p105 is often found in a cytoplasmic complex with c-Rel [33,34••], where its CTR is apparently acting *in trans* on the Rel polypeptide. The existence of this complex suggests that one IκB-like domain is sufficient to keep an entire NF-κB/rel dimer in the cytoplasm. Another function of the carboxyl termini may be to mask the nuclear localization signals in the RH domain, thus keeping the precursors or the p105/rel complex in the cytoplasm [17••,34••].

Proteolytic processing of the precursors, p105 and p100, into their active p50 and p52 forms, is unique to transcription factors of the NF-κB/rel family. Control of the still unidentified protease involved in processing is a likely locus of regulation. Pulse-chase studies have indicated that the CTR released by proteolysis is not stable and is rapidly degraded. When a p105-encoding plasmid is transfected into COS cells, the p105 is then slowly converted to p50 by cellular proteases. The enzyme involved and its regulation remain to be investigated [35]. HIV infection seems to be able to enhance p105 cleavage in infected cells, suggesting that processing could potentially be regulated by exogenous agents [36].

Why did proteolysis evolve as a mechanism of generating NF-κB activity? What is the advantage of processing? And, is processing a regulated process? There is a suggestion that the cytoplasmic retention of the precursor is crucial to keeping the NF-κB system from inappropriately stimulating cell growth. The *NFKB2* gene was recognized as the site of a specific chromosome translocation in a subset of human B- or T-cell tumors. Although the translocated gene has yet to be shown to be an oncogene by independent criteria, it probably is one, both because numerous tumors have a translocation at the same locus and because the p50 segment of *NFKB1* can be transforming under certain circumstances (GP Nolan, D Baltimore, unpublished data). In a panel of human tumors, the alterations of p100 are always such as to give constitutive nuclear localization of the p52-related fragment, i.e. they always lose the cytoplasmic retention domain at the carboxyl terminus (R Dalla-Favera, personal communication). Thus, proteolysis may normally be a way of releasing p52 when the cell needs it, with the precursor form being an inactive reservoir.

The other reason that p105 may be under processing control is to allow the cell to independently regulate two NF-κB/rel systems in the same cell. In B cells, it appears that p50/p65/IκB is regulated by phosphorylation, but the cell has cytoplasmic p105/c-Rel that can be proteolyzed to generate nuclear p50/c-Rel. These two types of NF-κB/rel proteins can thus co-exist and respond independently to different environmental stimuli or differentiation signals. Only when the processing pathway is better understood can these possibilities be critically examined.

Tissue-specific and developmental expression

Transcriptional control is a fundamental mechanism for gene regulation. Differential expression of certain members of NF-κB/rel and IκB genes in different tissues or during different stages of development will determine complex formation and subsequent target gene regulation (see next section). NF-κB (p50/p65) was identified as an apparently ubiquitous cytoplasmic factor early on; IκB was therefore expected to be widespread and later experiments bore out this prediction. However, some members of NF-κB/rel and IκB genes can be induced under certain circumstances. The genes that code for p105, p100 and IκB-α are highly inducible in response to the stimuli that activate NF-κB/rel activity in many cell types examined [6,37,38••,39]. The c-*rel* gene is expressed predominantly in lymphoid cells and can also be induced by the same stimuli that activate NF-κB/rel activity [37]. Thus, p105, p100, c-*rel* and IκB-α are considered as products of immediate response genes.

The regulation of the gene encoding IκB-α by NF-κB proteins was illustrated by introducing the genes encoding p50 or p65 into 3T3 cells followed by examining of the subcellular localization of the overexpressed products [40••]. Although both proteins contain apparent nuclear localization signals, p50 goes to the nucleus but p65 is mainly in the cytoplasm. Only if p65 is vastly overexpressed does a portion go to the nucleus. This differential behavior of the two NF-κB subunits can be traced to their effects on IκB-α: p65 induces IκB-α protein both by increasing the transcription of its gene and stabilizing the protein, while p50 has little effect on IκB-α. After co-expression of both p50 and p65, the protein are cytoplasmic, indicating that the induced IκB-α can maintain the entire NF-κB complex in the cytoplasm. The significance of the autoregulatory loop for NF-κB/rel and IκB system is that it allows a continuous maintenance of the cytoplasmic reservoir of NF-κB/rel complexes for further stimulation in an acute response, and also prepares the cell to return NF-κB to its uninduced condition afterwards.

NF-κB/rel activity is under complicated control during B-cell differentiation, which is just now becoming understood. In pre-B cells, NF-κB is maintained in an inactive form in the cytosol. The inactive complex is activatable by stimulation of pre-B cells with LPS or mitogens. By contrast, NF-κB-like activity is constitutively present in the nucleus of mature B cells. Studies on this constitutive NF-κB/rel activity suggest that it is predominantly a p50/c-Rel complex. Thus, in the same cells, there can be cytoplasmic p50/p65, which can be translocated to the nucleus after PMA induction, and nuclear p50/c-Rel (Table 3; H-C Liou, D Baltimore, unpublished data). Mature B cells contain much higher levels of p50 and c-Rel proteins than pre-B cells. As both the p105 and c-*rel* promoters contain κB sites (S Gerondakis, personal communication; [41,42]), transcriptional autoregulation by induced NF-κB may help to explain their relatively high level expression in B cells.

Except for mature B cells, most cells do not contain NF-κB/rel binding activity in un-induced conditions. Among the exceptions, KBF-1 (p50 homodimer) and p52 homodimers were detected in unstimulated T cells and macrophages by oligonucleotides containing the IL-2 or IL-2R κB sites (Table 3) [43••,44]. In T cells, stimulation of cells with PHA and PMA or infection with HTLV-1 Tax results in the appearance of two groups of complexes. The B1 complex contains p50 and p52 homodimers. The B2 complex consists of four proteins, p50, p52, p65 and c-Rel and probably represents multiple heterodimers of either p50 or p52 with either p65 or c-Rel [37,45]. In macrophages, the PMA- and TNF-α-induced complex is p50/p65 [44]. There is one report of a p65/c-Rel heterodimer that binds to the urokinase promoter in Hela cells induced with PMA [46].

In summary, the available data is still patchy despite this being an active area of research; it appears, however,

Table 3. NF-κB/rel protein complexes in EMSA.

	EMSA complexes				
Cell type	Constitutive	Treatment	Inducible	κB sites	Reference
B cell	p50/c-Rel (p50/p65)	PMA, LPS	p50/p65 (p50/c-Rel) $(p50)_2$ (by LPS)	Ig-κB	H-C Liou and D Baltimore, unpublished data; I Verma, personal communication
T cell		PMA, PHA	$(p65)_2$ p50/p65 p50/c-Rel	IL-2R	[45]
		HTLV	B1: $(p50)_2$ $(p52)_2$ B2: p50, p52 p65, c-Rel	IL-2R	[37]
	$(p50)_2$	Antigen	NF-κB-like	IL-2	[43••]
Macrophage	$(p50)_2$ $(p52)_2$	PMA, TNF	p50/p65	IL-2R	[44]
HeLa			LC: p50/p65 UC: p65/c-Rel	Urokinase	[46]

that the classic NF-κB, p50/p65, is the inducible factor in most cell types and responds to environmental stimuli by translocation from its stored cytoplasmic form to the nucleus. Other Rel-related factors, in particular c-Rel, can be constitutive κB-dependent activators, at least in cells of the B-cell lineage. It remains to be learned what signals trigger constitutive p50/c-Rel activity during B-cell differentiation.

The latest member of the family to be discovered is RelB. Recent studies by *in situ* hybridization analysis have suggested that RelB is expressed at particularly high levels in dendritic cells of the thymus. The strict expression of *relB* gene in dendritic cells may suggest a role related to antigen presentation (R Bravo, personal communication).

Combinatorial association and target gene specificity

Given the large number of possible Rel-related protein complexes, how much specificity of action exists? Specificity may be determined by both intrinsic differences between the properties of the complexes and differences in their interaction with other proteins. Although grouped into the same family, each individual NF-κB/rel protein or heterodimer complex may differ from other complexes in DNA-binding specificity, conformation upon binding to DNA, or transcription activity for a particular κB site. It is the subtle difference among various complexes of the same family that determines what target genes will be turned on in a given cell.

Apparently, not all of the so-far identified five members of the NF-κB/rel proteins can form heterodimers at a random combination. Among the 10 possible combinations, RelB can form a heterodimer with p50 or p52, but not with p65 or c-Rel (R Bravo, personal communication). The NF-κB/rel heterodimers have more or less broad binding specificity to various κB sites (Table 4). However, they show different properties when binding to different sites. NF-κB p50/p65 can bind to all the sites identified and serve as a transcription activator for all of them (Table 4) [43••,46,47•,48,49]. When p52/p65 was tested, it was found that it can also bind to a variety of sites but cannot activate reporter construct with either H2 or IL-2R κB sites (Table 4) [6,50,51]. Thus, p50 and p52 heterodimers have different effects on target genes despite the structural similarity between them. The transcriptional activities of p50–c-Rel, p52–c-Rel and p65–c-Rel have not been tested systematically.

The p50 homodimer (or KBF-1) binds at high affinity to most of the sites tested. Although p50 homodimer binds equally well to the Ig-κB, H2-κB and IFN-β-κB sites, it only transactivates expression from H2-κB and Ig/HIV κB sites [47•]. This is due to conformational differences of p50 when binding to these three sites. Therefore, conformation may determine whether a factor can act as an activator. The p52 homodimer differs from p50 in that it does not bind Ig/HIV-κB site, but binds well to the palindromic H2-κB site [5,50,51]. So far, the p52 homodimer has not been shown to activate transcription. The c-Rel homodimer was detected when certain κB sites were used, such as IL-2R-κB and IL-6-κB. Although c-Rel acts as a transcription activator for IL-2R, it seems to serve as a repressor for *IL-6* gene [49,52]. Again, it will be interesting to see if c-Rel binds to these two sites with a different conformation. There is one case where the endogenous p65 homodimer can be detected when the IL-2R-κB site was used [45]. The transcriptional activity of the p65 homodimer on the IL-2R site has not been addressed, although p65 is most likely to be an activator. The conclusion from this type of analysis is that each κB site preferentially binds a certain set of NF-κB/rel complexes as detected by EMSA. It is likely that these complexes may play major role in regulating target genes with this preferential κB site. Whether these complexes serve as activators or repressors will then be determined by their conformation on DNA and whether the transcription activation domains are accessible to transcription initiation machinery.

Interaction with non-Rel proteins

NF-κB/rel proteins interact with a variety of other proteins in cells. As discussed above, their interaction with the inhibitory IκBs (IκB-α, IκB-β and IκB-γ) results in inhibition of DNA-binding activity, masking of the nuclear localization signal and consequently cytoplasmic localization.

The interaction between p50 and Bcl-3, however, results in a more complicated and even controversial situation. It appears that Bcl-3 can be either an inhibitor of $(p50)_2$ binding to DNA or a coactivator that potentiates $(p50)_2$-mediated transcriptional activation (T Fujita *et al*, unpublished data) [13,18,53–56]. One group finds that it co-activates only $(p52)_2$-mediated transcription [53]. The fact that overexpressed Bcl-3 localizes to the nucleus distinguishes it from the other IκB proteins containing ankyrin repeats [18]. Also, the interaction between Bcl-3 and $(p50)_2$ has special properties because it does not require the NLS, whereas deletion of the NLS prevents IκB-α interaction with NF-κB [16••,56]. Studies of a bacterially produced, truncated version of Bcl-3, or of a baculovirus-produced Bcl-3, have shown that it can act as an *in vitro* inhibitor of DNA binding with preferential inhibitory affinity for p50 or p52 [18,53,54,56]. Using Bcl-3 protein made in mammalian cells, however, it was shown to activate *in vitro* transcription in a p50-dependent manner (T Fujita *et al*, unpublished data). With p50 or p52, it can also co-stimulate reporter gene transcription in mammalian cells (T Fujita *et al*, unpublished data) [57]. Bcl-3 is a highly phosphorylated protein in cells and dephosphorylation can inactivate its inhibitory activity [18]. Thus, part of the discrepancy in the behavior of the protein in different studies may be due to its phosphorylation state in different cells. An alternative hypothesis is that Bcl-3 can bind to $(p50)_2$ or $(p52)_2$ and both act as a co-activator and change the conformation of

[redacted]

Table 4. DNA-binding specificity and transcriptional activity of NF-κB/rel proteins

Complex	Binding sites*	Binding sites†	Transcriptional activation	Reference
p50/p65	IL-2 IL-2R IL-6 Urokinase	Ig/HIV IFN-β H2	Ig/HIV IFN-β H2	[43••,46] [47•,48] [49]
p52/p65		Ig/HIV PD H2 IL-2R	Ig/HIV (but not H2, IL-2R and PD)	[6,50] [51]
p50/RelB		Ig/HIV PD	Ig/HIV PD	[7]
p52/RelB		Ig/HIV PD	Ig/HIV (but not PD)	[6]
p52/p50		Ig/HIV PD	No activation	[6]
p50/c-Rel	Ig/HIV			(H-C Liou, D Baltimore, unpublished data) [45]
p52/c-Rel	IL-2R			[52]
p65/c-Rel	Urokinase			[46]
$(p50)_2$	Ig/HIV IL-2 IL-2R	Ig/HIV IFN-β H2	H2, Ig/HIV (but not IFN-β)	[43••,47•]
$(p52)_2$		H2 (but not Ig/HIV)	no activation	[5,50] [51]
$(p65)_2$	IL-2R	Ig/HIV IFN-β	IFN-β	[45,47•]
$(c\text{-}Rel)_2$		IL-2R IL-6 (but not Ig/HIV)	IL-2R repressor for IL-6	[49,52]
RelB	No homodimer			[7]

*Binding sites were tested by EMSA only; †binding sites were tested for both DNA binding and transcription activities. Transcription activities were determined mostly by transfection of reporter constructs with various κB sites. Studies by Fujita *et al.* were carried out both by transfection and *in vitro* transcription analyses. For κB site sequences, see Table 5.

their complexes with DNA so as to increase the off-rate of their binding to DNA. In this way, Bcl-3 could both apparently inhibit binding to DNA, as measured *in vitro*, and activate transcription during the time the complex is bound to DNA. Bcl-3 could also act as a stimulant to transcription because it could remove $(p50)_2$ from DNA and allow its replacement with the stronger activator p50/p65 as shown by Franzoso *et al.* [53].

NF–IL-6, a transcription factor of the C/EBP and bZIP family of proteins, has been shown to associate with p50 in solution [58]. The RH domain and the leucine-zipper motifs are important for this interaction. Although NF–IL-6 enhances p50 DNA binding activity, it is still unclear whether these two proteins can bind to DNA as a complex so as to synergistically activate transcription.

The transcriptional activator of HTLV-1, the Tax protein, can activate several cellular genes that also respond to NF-κB activation. It was recently shown that Tax protein can form a complex with p105 in HTLV-1 infected cells

Table 5. κB site sequences.

κB site	Sequence
Ig/HIV	GGGACTTTCC
H2	GGGATTCCCC
IFN-β	GGGAAATTCC
PD	GGGAATTCCC
IL-2	GGGATTTCAC
IL-2R	GGGAATCTCC
IL-6	GGGATTTTCC
Urokinase	GGGAAAGTAC

[59]. However, the significance of this association has yet to be determined.

NF-κB plays a critical role in virus induction of the *IFN-β* gene through the κB site in the PRDII region of the promoter. An accessory protein, HMG I(Y), has been found to cooperate with NF-κB activity by binding to the minor groove of the κB site [60••]. Both proteins are required for virus induction of *IFN-β* gene. It will be interesting to know how HMG I(Y) and NF-κB cooperate in the regulation of *IFN-β* gene.

Differentially spliced forms

Some genes of the NF-κB/rel family express differentially spliced RNAs that encode proteins with different properties. For example, IκB-γ is derived from the *NFKB1* locus that encodes p105. p65-delta is derived from the gene that encodes p65 and has 10 amino acid deletion in the dimerization domain. p65-delta protein losses its dimerization, DNA binding and transcription activities as well as the ability to stabilize IκB-α. The consequence of

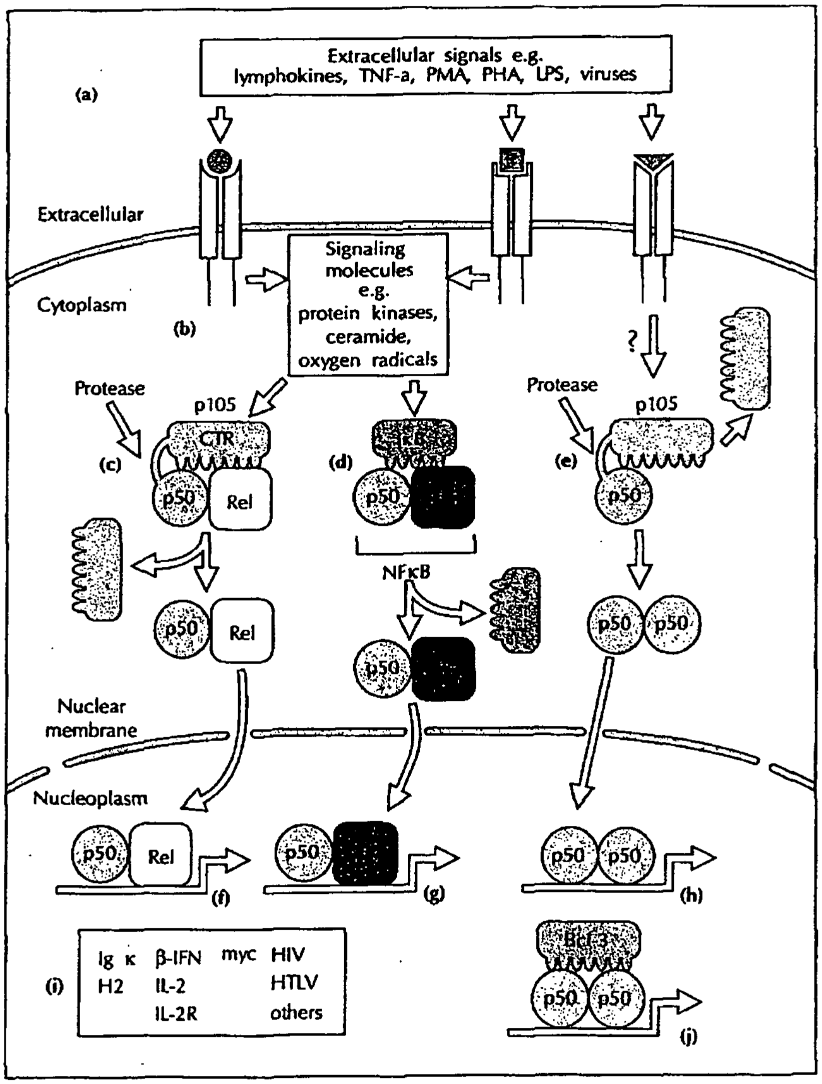

Fig. 1. Model of NF-κB/rel and IκB regulation. Signals received by various surface receptors (a) are transduced to NF-κB/rel and IκB complexes through either common or unique signaling molecules (b). The cytoplasmic NF-κB/rel complexes exist in two forms. One is a complex containing the precursors p105 (c) or p100 (although the p105 complexes are illustrated here, the p100 complexes are probably processed similarly). The other is a complex with an IκB (d). These two types of complexes may respond to different stimuli. p50 homodimers are generated by the processing of p105 (e) (p52 homodimers are similarly generated from p100; not shown). It is yet to be determined whether p105 (and p100) forms homodimers before processing occurs. As each NF-κB/rel complex differs from other complexes in DNA-binding specificity, conformation upon binding to DNA, or transcriptional activity for a particular κB site, various NF-κB/rel complexes may turn on different sets of target genes containing different κB sites (f)–(j). Specific target gene expression also depends on cooperation among NF-κB/rel proteins and other transcription factor complexes on the same promoter.

p65-delta expression in cells is still unclear, although its ability to transform cells has been suggested [61].

Prospects

Cytoplasmic/nuclear partitioning is central to the regulation of NF-κB/rel proteins (Fig. 1). The majority of the NF-κB/rel pool resides in the cytoplasm, either in complexes with uncleaved precursors or with separate IκBs. The cytoplasmic pool of NF-κB/rel protein can be rapidly activated after stimulation with immune cytokines or other regulators. The exact signal transduction pathways for activating NF-κB/rel are not understood yet, but studies of such regulators as TNF-α, *Toll* or oxygen radicals can be expected to shine light into these as-yet dark corners. In particular, what protein kinases are activated by ceramide upon TNF-α stimulation? How do Tube and Pelle proteins fit into the Toll and IL-1 pathways? What is the underlying mechanism for oxygen radical activation of NF-κB/rel activity?

The cytoplasmic complexes, as we now understand them, exist in two forms: one contains the p105 or p100 precursors; the other contains an IκB (Fig. 1). These two types of complexes may respond to different stimuli, thus providing diversity of regulation. Activating the precursor complexes must involve protease action but probably involves a second step because merely cleaving the precursor should not remove the inhibitor for the complex. It is also important to remember that we do not yet know whether processing of the precursor is a regulated process.

B cells (and perhaps the macrophages) are the only cell types where NF-κB/rel activity is constitutively present and active in the nucleus. This suggests that NF-κB/rel proteins carry out an important and special mission in the mature B cell. Part of that mission is certainly the expression of Ig κ light chain. It will be important to determine whether there are other key target genes in B cells because such genes could execute functions related to differentiation and proliferation. Another perplexing but intriguing question is what kinases or other signaling molecules are involved in inducing and maintaining the constitutive NF-κB/rel activity of B cells. Given the oncogenic potential of members of the NF-κB/rel protein family, it may also be relevant to ask whether the B cell has some special mechanism to avoid being transformed by constitutive NF-κB/rel proteins.

One of the most challenging questions is how alterations of the NF-κB/rel proteins cause tumors. One common phenomenon correlated to oncogensis caused by v-*rel* and p100 is their abnormal nuclear localization. Because both c-*rel* and p105 (and p100) play a major role in lymphoid cells, it may be reasonable that deregulation of these proteins causes predominantly lymphoid tumors. As both types of genes can undergo autoregulation, that might be an aspect of their tumorigenic abilities. It will be interesting to sort out the common features for tumorigenesis caused by v-*rel*, truncated p100 and *bcl*-3. The features to be examined will include their subcellular localization, autoregulation mechanism, and most importantly, their target genes in both normal and tumor cells.

Acknowledgments

We thank Drs M Scott, W Pear, Z-S Ye and B Sha for critical comments on this manuscript. We also appreciate Drs G Nolan, T Fujita, R Bravo, R Dalla-Favera, I Verma, S Wasserman and S Gerondakis among others for sharing information with us prior to publication.

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Cell, Vol. 58, 227–229, July 28, 1989, Copyright © 1989 by Cell Press

NF-κB: A Pleiotropic Mediator of Inducible and Tissue-Specific Gene Control

Minireview

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Individual regulators of cellular events often have different activities in specific cell types. For instance, cAMP mediates a vast array of responses through activation of protein kinase A because kinase substrates are distributed in specific patterns in different cell types. Transcriptional activation of specific genes in differentiated cells had been thought to require highly restricted expression of specific activator proteins. Increasingly, however, single transcriptional regulators, like other cellular regulators, have been found to have multiple effects, often depending on the cell type. A particularly promiscuous one, NF-κB, is so widespread that it poses the same regulatory questions as traditional second messengers such as cAMP and inositol polyphosphates. That is, how is specificity achieved and how do different pathways converge on the same regulatory molecule? Here we review how NF-κB works and the many systems known to employ it.

NF-κB was detected, using an electrophoretic mobility shift assay, as a protein that could complex to a 10 bp site in the κ light chain enhancer, called κB (Sen and Baltimore, 1986a). Because it was constitutively present only in those B cells of the appropriate stage for light chain expression and was crucial for κ enhancer function (Lenardo et al., 1987; Atchison and Perry, 1987), NF-κB appeared to be a tissue-restricted transcription factor. However, it was soon found to be present in a covert cytoplasmic form in non-B cells. In such cells it can be induced by phorbol esters to move to the nucleus and exhibit specific DNA binding. Consistent with its presence in all mammalian cell types tested, NF-κB has now been implicated in a variety of transcription events in non-B cells.

NF-κB Acts as an Intracellular Messenger

Induction of NF-κB does not require new protein synthesis. Indeed, inhibition of protein synthesis by cycloheximide can activate NF-κB (Sen and Baltimore, 1986b). This implies that NF-κB induction involves the conversion of a precursor into an active form. Recent experiments show that NF-κB is complexed with an inhibitory protein. Cytosolic extracts from uninduced cells can be treated in vitro with dissociating agents such as formamide and deoxycholate to unmask very high levels of NF-κB binding activity (Baeuerle and Baltimore, 1988a); such treatments generally do not work on nuclear extracts from uninduced cells. Conversely, activated NF-κB normally in cells is detected in nuclear but not cytosolic extracts, implying a nuclear translocation step associated with activation in vivo. A protein of about 68 kd is able to inhibit the DNA binding activity of NF-κB, but not other DNA binding proteins, and has therefore been named inhibitor-κB (IκB). Because IκB efficiently inhibits the activity of NF-κB derived from mature B cells or induced in vivo in other cell types, activation of NF-κB appears to involve a modification of IκB and not NF-κB (Baeuerle and Baltimore, 1988b). Complex formation between NF-κB and IκB is rapidly and efficiently reversible in vitro, which fits well with the finding that induction of NF-κB is reversible in vivo.

In a current model that ties together these observations, NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB. Various inducers then cause an as yet poorly understood alteration in IκB, allowing NF-κB to be released from the complex. NF-κB then travels to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription. This model implicates NF-κB directly in signal transduction; NF-κB, like the glucocorticoid receptor, acts as a messenger to transmit the gene induction signal from the cytoplasm to the nucleus.

The ability of phorbol esters to induce NF-κB suggests that IκB may be inactivated by a phosphorylation event involving protein kinase C. Recent evidence indicates that treatment of cytosol with protein kinase C in vitro releases NF-κB (Shirakawa and Mizel, 1989).

Constitutive NF-κB activity in B lymphocytes seems to result from regulation of either the level or activity of IκB. Certain mature B cell lines have significant NF-κB, but these levels increase further during viral induction (Lenardo et al., 1989). This observation argues against the possibility that transcription of IκB is simply shut off at the final stages of B cell maturation. Instead, B cell differentiation may be associated with partial, constitutive NF-κB activity, while additional NF-κB remains complexed with IκB to serve inducible gene functions. The molecular features of NF-κB activation, especially the independence from new protein synthesis, embody an efficient system for rapidly transducing extracellular signals into specific patterns of gene expression in the nucleus.

NF-κB Takes Part in T Cell Activation

The inducibility of NF-κB in a variety of cell types suggested that it plays a role in other genes besides the immunoglobulin κ gene. This was shown in studies of the interleukin-2 α receptor (IL-2Rα) gene, which is expressed during activation of T lymphocytes. Transcriptional stimulation of the IL-2Rα gene is partly governed by the binding of NF-κB to a site in the IL-2Rα promoter (Böhnlein et al., 1988). NF-κB is induced by several T cell mitogens and by antibodies against cell surface markers that mimic physiologic T cell activation (Sen and Baltimore, 1986b; Tong-Starksen et al., 1989). NF-κB is also one of the signals for inducing the IL-2 lymphokine gene itself (Shibuya et al., 1989). Thus NF-κB appears to be a vital control element in T cell activation.

Significantly, the *trans*-activator gene product (*tax*) of the human T lymphotropic virus HTLV-I powerfully induces NF-κB (Cross et al., 1989). One can envisage that early during HTLV-I infection, abnormal induction of NF-κB by *tax* causes unregulated T cell growth. Such proliferation would predispose the infected T lymphocytes to a second transforming mutation, leading to frank leukemia. The connection between HTLV-I and leukemogenic events in T cells appears to represent a subversion of the normal activation of NF-κB that occurs during the immune response.

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NF-κB Is an Important Participant in Cytokine Regulation

Cytokines are proteins that convey signals for homeostatic regulation of cellular proliferation, differentiation, and other activities (for review see DeMaeyer and DeMaeyer-Guignard, 1988). NF-κB seems to be ideally suited for the regulation of intercellular signals. It can act as a transient signal when induced by phorbol esters, or as a prolonged signal when induced by lipopolysaccharide (Sen and Baltimore, 1989b). In addition to taking part in the induction of IL-2, NF-κB has been found to positively regulate the human β-interferon (β-IFN) gene: it interacts with a virus-inducible element (PRDII) in the β-IFN gene and is highly induced by virus infection or treatment of cells with double-stranded RNA (Visvanathan and Goodbourn, 1989; Lenardo et al., 1989). It is important to note that β-IFN gene expression can be elicited in many cell types other than lymphocytes, which reinforces the idea that there is nothing intrinsically tissue specific about NF-κB activation.

NF-κB has also been shown to take part in the cytokine-mediated elevation in certain plasma proteins in the liver in response to inflammation (Edbrooke et al., 1989). NF-κB is likely to play a role in the expression of genes for other cytokines such as IL-6, TNF-α, and lymphotoxin. Moreover, NF-κB can itself be induced by TNF-α and IL-1 (Osborn et al., 1989), revealing an interesting mechanism for autostimulatory and co-stimulatory regulatory loops.

Viruses Use NF-κB

Viruses other than HTLV-I have taken direct advantage of NF-κB's powerful transcriptional effect. Both SV-40 and cytomegalovirus (CMV) have NF-κB binding sites in their enhancers, and viral *trans*-activators of CMV and hepatitis B virus induce NF-κB activity (Sen and Baltimore, 1986a; Cherrington and Mocarski, 1989; Siddiqui et al., 1989). Most importantly, the enhancer of human immunodeficiency virus type 1 (HIV-1) has two prominent NF-κB binding sites (Muesing et al., 1987). NF-κB binding regulates the transcriptional inducibility of the HIV long terminal repeat (LTR) in activated T lymphocytes or phorbol ester–stimulated HeLa cells (Nabel and Baltimore, 1987). Thus, latency of the HIV provirus due to a lack of LTR-mediated transcription in resting T cells could be overcome by induction of NF-κB upon activation of T cells, resulting in elevated transcription of the provirus and increased virus production. Support for this model comes from evidence that viral production is stimulated by agents that induce NF-κB in monocytic cell lines bearing an HIV provirus (Griffin et al., 1989).

Specificity Requires Additional Factors

How is "cross talk" avoided between the various pathways employing NF-κB? Given the ubiquity of systems using NF-κB, a level of regulation in addition to NF-κB induction is needed to ensure that each signal gives rise to a specific response. Investigations of NF-κB–regulated genes have defined other transcription factors that govern the response to the NF-κB signal. A clear example comes from studies of β-IFN gene regulation: viral induction of β-IFN is mediated by two virus-inducible activators, one of which is NF-κB, and the release of a repressor (Maniatis, 1988; Lenardo et al., 1989). The two activators bind distinct DNA sites (PRDI and PRDII) but must act together to respond to induction. As single copies, neither site is inducible (Fan and Maniatis, 1989).

The theme of multiple signals that generate specificity is supported by studies of the immunoglobulin κ and IL-2Rα genes. The NF-κB site from the κ light chain enhancer alone stimulates transcription in B and T lymphocytes as well as in nonlymphoid cells. By contrast, the entire κ enhancer is inducible only in B lymphocytes and is unresponsive to NF-κB in other cell types (Pierce et al., 1988). The restricted response to NF-κB by the κ enhancer has now been attributed to a silencer sequence that resides in the enhancer some distance away from the NF-κB binding site and acts to suppress transcriptional effects of NF-κB in non-B cells. Specific activation of the IL-2Rα gene in T lymphocytes is attained by slightly different means: full induction of this gene depends on NF-κB as well as a positively acting sequence immediately downstream (Cross et al., 1989).

Is There One NF-κB?

Similar sequences in a variety of genes are recognized by a phorbol ester–induced protein that forms a DNA–protein complex indistinguishable in size from that formed by NF-κB (see table). Sites from genes induced in distinct cell types often differ slightly in sequence. Could the appearance of a single factor belie a family of closely related but functionally distinct transcriptional regulators? By this scheme, one member of the family could be the constitutive factor in B cells, while others could be specifically inducible in particular cell types.

The hypothesis of multiple, similar NF-κB species has not yet been tested directly, but biochemical evidence does not suggest heterogeneity. No differences in binding specificity have been detected between the NF-κB proteins from different cell types. That is, the NF-κB protein induced in T cells has no preference for sites from genes activated in T cells over those from genes activated in B cells, and vice versa (Lenardo et al., 1988). Furthermore, the complexes between DNA and NF-κB from different cell types involve an identical pattern of base contacts.

The biochemical features of the NF-κB complex are the same regardless of the cell type from which it is derived. Purification of NF-κB to homogeneity from human cells yields a single DNA binding polypeptide chain of approximately 50 kd (Kawakami et al., 1988). An associated 65 kd polypeptide may be an intrinsic part of the NF-κB complex. NF-κB has the unique property that nucleoside

Table 1. Sequences Recognized by NF-κB

Gene	Sequence
Mouse Ig κ enhancer SV40 enhancer HIV-1 (−91) CMV (4)	(G)GGGACTTTCC
HIV-1 (−105) HIV-2 CMV (1) β_2-microglobulin Serum amyloid A	(A)GGGACTTTCC
Human Ig κ enhancer CMV (3)	GGGGATTTCC
β-IFN (PRDII)	GGGAAATTCC
CMV (2)	GGGACTTTCC
MHC class II (Eα^d)	GGGACTTCCC
IL-2	GGGATTTCAC
Mouse IL-2Rα	GGGGATTCCT
Human IL-Rα	GGGAATCTCC
MHC class I (H-2K^b) HLA (A2, A11, B7, B27, B51)	GGGATTCCCC
Consensus:[a]	C GGGRATYYCC T

The binding nucleotides are identical for the first two classes of sites, but the 5′ border nucleotide (in parentheses) is conserved as either an A or G; the regulatory significance of this is not known. The four putative NF-κB recognition sites in the CMV enhancer (numbered 1–4, as they are found from 5′ to 3′ on the coding strand) have not been tested in a binding assay. All others have been proven by direct binding, and usually also by inhibition of binding to the immunoglobulin κ sequence.

Data are from Sen and Baltimore (1986a), Nabel and Baltimore (1987), Gruyader et al. (Nature *326*, 662–669, 1987), Yano et al. (EMBO J. *6*, 3317–3324, 1987), Edbrooke et al. (1989), Emorine et al. (PNAS *81*, 1789–1793, 1984), Lenardo et al. (1989), Visvanathan and Goodbourn (1989), Blanar et al. (Mol. Cell. Biol. *9*, 844–846, 1989), Lenardo et al. (1988), Cross et al. (1989), Böhnlein et al. (1988), Baldwin and Sharp (1988), and Hakem et al. (J. Immunol. *142*, 297–305, 1989).

[a] Based on all sequences, but the assignments of the sixth, ninth, and tenth positions ignore one deviant.

triphosphates dramatically stimulate its ability to bind DNA in vitro (Lenardo et al., 1988); and, as mentioned above, NF-κB can be released as an active species from an inactive cytosolic form (Baeuerle and Baltimore, 1988a).

It is clear that NF-κB binding sites are also recognized by other, obviously distinct transcription factors. One example is the H2TF1 protein, which binds to an NF-κB–like site in the mouse H-2K^b class I major histocompatibility gene (Baldwin and Sharp, 1988). This factor, however, is a constitutively active nuclear binding protein in many different cell types and is not implicated in inducible gene expression.

Conclusion

Taken together, these findings indicate that there is probably only one NF-κB or perhaps a closely related protein family that serves multiple roles in many different cell types. Understanding the properties of NF-κB will provide an explanation for how nature uses a single transcription factor to operate a diversity of gene induction processes.

A detailed understanding of the regulation of NF-κB will be greatly facilitated by the cloning of its gene. NF-κB is present in very low abundance, and has thus far confounded conventional cloning techniques (Lenardo et al., 1988). Equally important in future work will be a thorough biochemical investigation of the dissociation of NF-κB and IκB in the cell. No doubt, NF-κB will continue to yield a fascinating look into the underlying logic of transcriptional regulation.

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